University of Alberta

Immunity of Goldfish *Carassius auratus* (L). to *Trypanosoma danilewskyi* Laveran and Mesnil, 1904

by

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ABSTRACT

Trypanosoma danilewskyi is a protozoan parasite of different species of freshwater fishes. Previous research has established that immunity to *T. danilewskyi* is antibody mediated, involving development of immunological memory. Researchers from our laboratory have shown that immunization of goldfish with excretory-secretory (ES) products of *T. danilewskyi* induced protective immunity to homologous challenge. The objective of this thesis was to further characterize the protective immune response of goldfish to *T. danilewskyi* infection.

The specific aims of this research were: (1) to identify specific antigenic component(s) of *T. danilewskyi* ES products; (2) to sequence, clone, and express the genes encoding the antigenic ES molecule(s) in a prokaryotic expression system for use in immunization studies; and (3) to evaluate the role of goldfish complement in antibody-mediated immunity.

Trypanosome tubulin was identified as a component of ES products that stimulated antibody production in goldfish. The nucleotide sequences for the *T. danilewskyi* α- and β-tubulin subunits were determined and the proteins were expressed in *Escherichia coli*. Rabbit-anti-recombinant α-tubulin IgG, and IgM purified from naïve and infected goldfish prevented the growth of parasites *in vitro* by a mechanism that may involve internalization of the antibodies. Immunization of goldfish with endotoxin-free recombinant proteins, in conjunction with Freund's complete adjuvant, resulted in a significant decrease in the average parasitemia and prevalence of infection within the first week after inoculation of parasites. It was also observed that non-immunized and non-infected goldfish have antibodies that

recognize trypanosome tubulin, suggesting a role for natural antibodies in the immunity of goldfish to infection.

In vitro cultured trypanosomes were resistant to lysis by goldfish complement regardless of the presence of specific antibodies. Resistance was related to the ability of trypanosomes to synthesize protective surface proteins because trypsin treatment of the parasites rendered them susceptible to lysis by the alternative pathway of complement activation.

Taken together, these results indicate that the protective immune response of goldfish against *Trypanosoma danilewskyi* was in part controlled by both specific as well as non-specific host defense molecules.

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TABLE OF CONTENTS

Chapter 1. General Introduction and Objectives.. 1

1.1 Introduction.. 1

Objectives.. 3

Chapter 2. Literature Review.. 4

2.1 Introduction.. 4

2.2 Comparison of the immune response of mammals and fish to trypanosome infection.. 6

2.2.1 Soluble mediators of immunity.. 7

2.2.1.1 The acute phase response.. 7

2.2.1.2 Trypanolytic factors.. 11

2.2.1.3 Cytokines.. 16

2.2.1.4 Antimicrobial peptides.. 19

2.2.1.5 Complement.. 24

2.2.1.6 Antibody responses.. 31

2.2.1.6.1 Natural antibodies.. 31

2.2.1.6.2 Specific antibodies.. 34

2.2.2 Cellular mediators of immunity.. 36

2.2.2.1 Innate immune response.. 36

2.2.2.1.1 Pattern recognition receptors.. 37

2.2.2.1.2 Effector cells.. 40

Macrophages.. 40

Classical versus alternative macrophage activation.. 40

Phagocytosis.. 44

NK cells.. 45

Non-specific cytotoxic cells.. 46

Cytotoxic NK-like cells.. 47

2.2.2.2 Acquired immune response.. 51

2.2.2.2.1 T-cell responses.. 52

NKT cells.. 56

2.2.2.2.2 B-cell responses........................... 58

2.3 Conclusion.. 62

Chapter 3. Materials and Methods.. 64

3.1 General Procedures.. 64

3.1.1 Fish.. 64

3.1.2 Bleeding of fish and preparation of fish serum/plasma..... 64

3.1.3 Parasites.. 64

3.1.4 Purification of parasites from the blood......................... 65

3.1.5 Infection of fish.. 65

3.1.6 Assessment of infection... 65

3.1.7 Antigen preparation.. 66

3.1.7.1 Whole cell lysates.. 66

3.1.7.2 Excretory-secretory (ES) products...................... 66

3.1.7.2.1 Fractionation of ES products using ion exchange chromatography (Mono-Q)................. 66

3.1.7.3 Detergent soluble molecules.............................. 67

3.1.8 Determination of packed red cell volume...................... 67

3.1.9 Anti-carp IgM antibody.. 68

3.1.10 SDS-PAGE and Western blotting............................. 68

3.1.10.1 SDS-PAGE.. 68

3.1.10.2 Western blotting.. 68

3.1.11 Estimation of protein concentration............................ 69

3.1.12 Production of polyclonal rabbit antibodies.................... 69

3.1.13 Isolation of polyclonal IgG from rabbit serum................ 70

3.1.14 Mass spectrometry.. 70

3.2 Isolation of *T. danilewskyi* α-and β-tubulin cDNA sequences and production of recombinant proteins.. 71

3.2.1 cDNA synthesis and RT-PCR..................................... 71

3.2.2 *In silico* analysis... 71

3.2.3 Cloning and expression of recombinant trypanosome tubulin subunits.. 72

3.2.4 Scale-up expression and isolation of recombinant trypanosome tubulin subunits........ 73

3.2.5 Purification of recombinant proteins........ 75

3.2.5.1 Nickel-agarose chromatography........ 75

3.2.5.2 Magnetic separation........ 76

3.2.6 Endotoxin removal........ 77

3.2.7 Indirect immunofluorescence........ 77

3.3 Effects of rabbit and goldfish antibodies on trypanosome growth *in vitro* and immunization of goldfish with recombinant α- and β-tubulin subunits........ 78

3.3.1 The effects of rabbit-anti-recombinant α-tubulin polyclonal antibody on trypanosome growth *in vitro*........ 78

3.3.2 Purification of goldfish serum IgM........ 78

3.3.3 The effects of goldfish IgM on the growth of *T. danilewskyi in vitro*........ 79

3.3.4 Immunization of goldfish........ 80

3.4 Effects of goldfish complement on *T. danilewskyi in vitro*........ 80

3.4.1 Enzymes and inhibitors........ 80

3.4.2 Enzymatic treatment of trypanosomes........ 80

3.4.3 Assay for trypanosome lysis........ 81

3.4.4 Chelation and re-supplementation of divalent cations........ 81

3.4.5 Restoration of resistance........ 81

3.5 Attempted identification of a *T. danilewskyi* complement-binding molecule........ 82

3.5.1 Purification of goldfish complement component three...... 82

3.5.1.1 Rabbit-anti-carp C3 immunoglobulin........ 82

3.5.1.2 Polyethyleneglycol (PEG) precipitation of goldfish serum........ 82

3.5.1.3 Anion exchange using Q-sepharose chromatography........ 83

3.5.1.4 Gel-permeation fast performance liquid chromatography........ 83

3.5.1.5 Anion exchange using Mono-Q........ 84

3.5.2 Affinity chromatography using goldfish C3........................ 84
3.5.3 Far-Western blotting... 85
3.5.4 Construction of a *T. danilewskyi* cDNA library.................. 85
3.5.5 Screening of the trypanosome cDNA library using a *T. danilewskyi* sialidase domain probe.. 86
3.6 Statistics.. 87

Chapter 4. Description of the course of infection................................ 91
4.1 Introduction.. 91
4.2 Experimental design.. 91
4.3 Results... 92
4.4 Discussion.. 93

Chapter 5. Identification of *T. danilewskyi* tubulin in parasite excretory-secretory products and expression of recombinant parasite tubulin subunits...... 99
5.1 Introduction.. 99
5.2 Experimental design.. 100
5.2.1 Identification of the antigenic component(s) of *T. danilewskyi* excretory-secretory products.............................. 100
5.2.2 Amplification of *T. danilewskyi* α- and β-tubulin gene sequences, cloning, and expression of recombinant proteins...... 100
5.3 Results... 101
5.3.1 Identification of tubulin in *T. danilewskyi* ES products...... 101
5.3.2 Identification of *T. danilewskyi* α- and β-tubulin cDNA sequences... 101
5.3.3 Cloning of *T. danilewskyi* α- and β-tubulin into pET 100/D-TOPO vector... 102
5.3.4 Recognition of native and recombinant α- and β-tubulin by polyclonal rabbit anti-serum.. 103
5.3.5 Indirect immunofluorescence.. 104
5.4 Discussion.. 104

Chapter 6. Immunization of goldfish with recombinant *T. danilewskyi* α- and β-tubulin and the effects of rabbit anti-tubulin and goldfish antibodies on parasite growth *in vitro*........ 120

6.1 Introduction........ 120

6.2 Experimental design........ 121

6.2.1 Immunization of goldfish with recombinant *T. danilewskyi* α- and β-tubulin........ 121

6.2.2 The effect of rabbit-anti-recombinant α-tubulin IgG on parasite growth *in vitro*........ 123

6.2.3 The effect of goldfish IgM from naïve and trypanosome infected goldfish on the growth of *T. danilewskyi in vitro*........ 124

6.3 Results........ 125

6.3.1 Immunization of goldfish with parasite α- and β-tubulin subunits increases their resistance to *T. danilewskyi* infection.... 125

6.3.1.1 Immunization with endotoxin-free recombinant tubulin subunits........ 125

6.3.1.2 Immunization with recombinant tubulin subunits containing LPS........ 128

6.3.2 Inhibition of parasite growth *in vitro* by anti-recombinant α-tubulin IgG........ 130

6.3.3 Purification of goldfish IgM and inhibition of trypanosome growth *in vitro* by PEG precipitated and superose 6 purified goldfish IgM........ 131

6.4 Discussion........ 133

Chapter 7. The effect of goldfish complement on *T. danilewskyi in vitro*....... 155

7.1 Introduction........ 155

7.2 Experimental design........ 156

7.2.1 Determination of the susceptibility of *in vitro* cultivated trypanosomes to lysis in serum from naïve and recovered hosts........ 156

7.2.2 Enzyme treatment of *in vitro* cultured trypanosomes........ 157

7.2.3 Determination of the pathways of complement activation........ 158

7.2.4 Restoration of trypanosome resistance to complement mediated lysis........ 158

7.3 Results.......... 159
    7.3.1 Susceptibility of normal and enzyme treated *T. danilewskyi* to lysis by goldfish serum.......... 159
    7.3.2 Effect of removal of divalent cations from goldfish serum on the susceptibility of trypsinized trypanosomes to lysis.......... 160
    7.3.3 Restoration of resistance to lysis of trypsin-treated trypanosomes.......... 161
7.4 Discussion.......... 161

Chapter 8. Purification of goldfish complement component three (C3) in an attempt to identify a *T. danilewskyi* complement-binding protein.......... 174
8.1 Introduction.......... 174
8.2 Experimental design.......... 176
    8.2.1 Purification of goldfish C3.......... 176
        8.2.1.1 C3b-affinity chromatography.......... 177
        8.2.1.2 Far-Western blotting.......... 177
    8.2.2 RT-PCR using primers designed from the *T. cruzi* CRP and screening of a *T. danilewskyi* cDNA library.......... 178
8.3 Results.......... 178
    8.3.1 Purification of goldfish C3 from serum.......... 178
    8.3.2 C3b affinity chromatography.......... 179
    8.3.3 Far-Western blotting.......... 179
    8.3.4 RT-PCR using primers designed from the *T. cruzi* CRP and screening of a *T. danilewskyi* cDNA library.......... 179
8.4 Discussion.......... 180

Chapter 9. General discussion.......... 187
9.1 Overview of findings.......... 187
9.2 Future directions.......... 198
9.3 Conclusion.......... 201
References.......... 203

LIST OF TABLES

Table 3-1. Composition of *Trypanosoma danilewskyi* culture medium.............. 88

Table 3-2. List of primers.. 89

Table 6-1. Summary of immunization trials... 142

Table 7-1. Lysis of trypsin-treated *Trypanosoma danilewskyi* by sera from infected and uninfected goldfish.. 166

Table 7-2. Lysis of trypsin-treated *Trypanosoma danilewskyi* by non-immune goldfish serum at low temperature.. 167

Table 7-3. The effect of *Arthrobacter ureafaciens* sialidase treatment on the susceptibility of *Trypanosoma danilewskyi* to lysis by normal and sialidase treated non-immune goldfish serum.. 168

Table 7-4. The effect of *Clostridium perfringens* sialidase treatment on the susceptibility of *Trypanosoma danilewskyi* to lysis by normal and sialidase treated non-immune goldfish serum.. 169

Table 7-5. The effects of Mg^{2+} chelation on the ability of non-immune goldfish serum to cause lysis of trypsin treated *Trypanosoma danilewskyi*....................... 170

Table 7-6. The effect of Ca^{2+} chelation on the ability of non-immune goldfish serum to cause lysis of trypsin treated *Trypanosoma danilewskyi*....................... 171

LIST OF FIGURES

Figure 3-1. Purified rabbit-anti-carp complement component three (C3) IgG cross-reacts with C3 in goldfish serum.......... 90

Figure 4-1. Wright's-stained smear of *in vitro* cultured *T. danilewskyi*.......... 96

Figure 4-2. Course of infection, prevalence, mortality, and volume of red blood cells in naïve goldfish inoculated with *Trypanosoma danilewskyi*.......... 97

Figure 4-3. *In vitro* growth of *Trypanosoma danilewskyi* in medium supplemented with 10% heat-inactivated goldfish serum.......... 98

Figure 5-1. Identification of molecules contained in the excretory/secretory (ES) products of *Trypanosoma danilewskyi* that induce antibody production in infected goldfish.......... 110

Figure 5-2. The deduced nucleotide sequence of *Trypanosoma danilewskyi* α-tubulin.......... 111

Figure 5-3. Amino acid alignment of the predicted *Trypanosoma danilewskyi* α-tubulin sequence.......... 112

Figure 5-4. The deduced nucleotide sequence of *Trypanosoma danilewskyi* β-tubulin.......... 113

Figure 5-5. Amino acid alignment of the predicted *Trypanosoma danilewskyi* β-tubulin sequence.......... 114

Figure 5-6. Amplification of *Trypanosoma danilewskyi* α- and β-tubulin gene products by RT-PCR for cloning into the pET100D TOPO prokaryotic expression vector.......... 115

Figure 5-7. Detection of the expression of recombinant α-and β-tubulin in *E. coli* cell lysates.......... 116

Figure 5-8. Reactivity of rabbit-anti-recombinant α-tubulin IgG with native *Trypanosoma danilewskyi* tubulin.......... 117

Figure 5-9. Immunofluorescence assay using anti-recombinant α-tubulin IgG and permeabilized *Trypanosoma danilewskyi*.......... 118

Figure 5-10. Immunofluorescence assay using anti-recombinant α-tubulin IgG and non-permeabilized *Trypanosoma danilewskyi*.......... 119

Figure 6-1. The effect of intraperitoneal administration of 20 μg of detoxified recombinant α-tubulin + FCA or control solution to goldfish prior to infection with *T. danilewskyi*.......... 143

Figure 6-2. The effect of intraperitoneal administration of 40 μg of endotoxin-free recombinant α-tubulin+ FCA or control solution to goldfish prior to infection with *T. danilewskyi*.......... 144

Figure 6-3. The effect of intraperitoneal administration of 20 μg of endotoxin-free recombinant β-tubulin + FCA or control solution to goldfish prior to infection with *T. danilewskyi*.......... 145

Figure 6-4. The effect of intraperitoneal administration of 40 μg of endotoxin-free recombinant β-tubulin + FCA or control solution to goldfish prior to infection with *T.danilewskyi*.......... 146

Figure 6-5. Goldfish immunized with endotoxin-free recombinant *T. danilewskyi* α-and β-tubulin have antibodies that recognize the immunizing antigen.......... 147

Figure 6-6. The effect of intraperitoneal administration of 20 μg and 40 μg of recombinant α-tubulin containing LPS + FCA or control solution to goldfish prior to infection with *T. danilewskyi*.......... 148

Figure 6-7. The effect of intraperitoneal administration of 20 μg and 40 μg of recombinant β-tubulin containing LPS + FCA or control solution to goldfish prior to infection with *T. danilewskyi*.......... 149

Figure 6-8. Goldfish immunized with recombinant *T. danilewskyi* α-and β-tubulin containing endotoxin have antibodies that recognize the immunizing antigen.......... 150

Figure 6-9. The effects of affinity-purified anti-α-tubulin IgG on growth of *Trypanosoma danilewskyi in vitro*.......... 151

Figure 6-10. Purification of goldfish IgM using polyethylene glycol 6000 precipitation and gel permeation chromatography.......... 152

Figure 6-11. The effects of partially purified goldfish IgM on the growth of *T. danilewskyi in vitro*.......... 153

Figure 6-12. The effects of purified goldfish IgM and pre-absorbed purified goldfish IgM on the growth of *T. danilewskyi in vitro*.......... 154

Figure 7-1. Effect of serum dilution on lysis of two different concentrations of trypanosomes.......... 172

Figure 7-2. Ability of trypsinized trypanosomes to regain resistance to lysis by goldfish serum.. 173

Figure 8-1. Polyethylene glycol precipitation of goldfish serum for isolation of goldfish complement component three (C3).. 183

Figure 8-2. Polyethylene glycol precipitated goldfish C3 separated by anion exchange chromatography.. 184

Figure 8-3. Gel permeation fast performance liquid chromatography (GP-FPLC) separation of the goldfish C3-containing fractions isolated by anion exchange using the Hitrap Q-FF column.. 185

Figure 8-4. Final purification of goldfish C3 by anion exchange using a Mono-Q column.. 186

LIST OF ABBREVIATIONS

ANOVA-analysis of variance
APC-antigen presenting cell
APP-acute phase protein
APR-acute phase reaction
BCIP-5-bromo-4-chloro-3-indolyl phosphate
BCR-B-cell receptor
bps-base pairs
BSA-bovine serum albumin
cDNA-complementary DNA
CRP-complement regulatory protein
DC-dendritic cell
DIC-differential interference contrast
DNA-deoxyribonucleic acid
EACA-6-aminocaproic acid
EDTA- ethylenediaminetetraacetic acid
EGTA- ethylenebis(oxyethylenenitrilo)tetraacetic acid
ELISA-enzyme-linked immunosorbent assay
ES-excretory/secretory
EU-endotoxin units
FCA-Freund's complete adjuvant
FIA-Freund's incomplete adjuvant
FITC-fluorescein isothiocyanate
GFS-goldfish serum
GIPL-glycoinositolphospholipid
GP-FPLC-gel permeation fast performance liquid chromatography
GPI- glycophospatidylinositol
GSH-reduced glutathione
GSSG-oxidized glutathione
HDL-high density lipoprotein
HI-heat-inactivated
Hpr-haptoglobin-related protein
IFN-interferon
IgG-immunoglobulin G
IgM-immunoglobulin M
IL-interleukin
iNOS-inducible nitric oxide synthase
IP-intraperitoneal
IPTG-isopropyl-beta-D-thiogalactopyranoside
kDa-kiloDalton
LAL-Limulus amebocyte lysate
LB-Luria-Bertani
LPS-lipopolysaccharide
MAC-membrane attack complex
MASP-MBL-associated serine protease

MBL- mannose-binding lectin
MMLV-RT-Moloney murine leukemia virus reverse transcriptase
MWCO-molecular weight cutoff
NBT-nitro blue tetrazolium
NCC-natural cytotoxic cell
NCCRP-1-natural cytotoxic cell receptor protein-1
NK-natural killer
NKT-natural killer T-cell
NO-nitric oxide
OD-optical density
PAMP-pathogen associated molecular pattern
PBS-phosphate buffered saline
PCR-polymerase chain reaction
PEG-polyethyleneglycol
PMSF- phenylmethylsulfonylfluoride
RAG- recombination activating gene
RNA-ribonucleic acid
RT-PCR-reverse-transcriptase-PCR
SBTI-soybean trypsin inhibitor
SDS-PAGE-sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM-standard error of the mean
SRA-serum resistance-associated gene
TBS-tris-buffered saline
TCR-T-cell receptor
TGF-transforming growth factor
Th1/2-T-helper cell
TLF-trypanosome lytic factor
TLR-toll-like receptor
TLTF-trypanosome lymphocyte triggering factor
TNF-tumor necrosis factor
TTBS-TBS+Tween 20
VSG-variable surface glycoprotein

CHAPTER 1

GENERAL INTRODUCTION AND OBJECTIVES

1.1 Introduction:

The experimental model system of *Trypanosoma danilewskyi* infection in goldfish is representative of a natural protozoan parasite infection in a lower vertebrate host. These flagellated unicellular organisms live extracellulary in the bloodstream of freshwater fish and are commonly found in natural and farmed fish populations [218]. *T. danilewskyi* is a member of the genus *Trypanosoma*, which also includes the species *T. brucei* and *T. cruzi* that infect a variety of mammals, including humans.

Despite the medical and veterinary importance of the mammalian trypanosomes as disease causing agents, *T. danilewskyi* is considered to be non-pathogenic in fish. While this may be true in natural fish populations, it seems that the 'non-pathogenicity' of the parasite may actually be related to the ability of an immunocompetent host to control the infection. Support for this hypothesis can be found in the reports of high prevalence of the infection in aquaculture settings. High population densities and animal handling contribute to increased stress, and renders an otherwise healthy fish unable to prevent parasite-induced pathogenesis [283].

Although mortality may not be directly related to parasitemia, immunosuppression is a characteristic feature of trypanosome infections in all vertebrates and immunocompromised individuals often succumb to secondary infections. As such, the study of the fish response to trypanosome infection presents an excellent opportunity to examine the nature of protective immunity to blood-borne protozoan infections. The majority of the studies involving the control of trypanosomes infecting mammals have been done in mice, which are not natural hosts for these pathogens. The *T. danilewskyi* infections in goldfish may provide a better model for the study of the vertebrate-trypanosome relationship from an evolutionary perspective. In fact the value of studying the immune responses of phylogenetically 'primitive' organisms to the understanding of the regulation of mammalian defenses has become better appreciated in the past few years [172, 240].

Experimental infections of goldfish (*Carassius auratus*) with *T. danilewskyi* can result in clinical disease and occasionally mortality in fish harbouring high numbers of parasites [170, 390, 403]. The symptoms of infection include anemia and anorexia, which can lead to death of the host [168-9]. The immune responses of fish to *T. danilewskyi* infection have not been well studied despite the fact that the hosts are easily, and inexpensively, maintained compared to the requirements of the model hosts for mammalian trypanosome infections. In addition, *T. danilewskyi* can be cultured *in vitro* providing the opportunity to grow large numbers of parasites for molecular and biochemical analyses [41, 284].

The prevailing opinion, based on the results of a small number of studies, suggests that specific antibody responses are critical for the control of *T. danilewskyi* infections in carp (*Cyprinus carpio*) and goldfish. The evidence to support this conclusion includes: (1) the resistance of recovered fish to re-infection [40, 170, 390]; (2) the passive transfer of immunity to naïve hosts by injection of serum or purified IgM from recovered hosts [283, 403]; (3) the suppression of protective immunity to re-infection in recovered fish by administration of corticosteroids [170]; and (4) the production of antibodies in infected carp that recognize parasite cell lysates in an ELISA [283]. Although few studies have determined the roles of non-specific and cell-mediated responses to *T. danilewskyi* infections it has been suggested that antibody and cell-mediated responses may be involved in the protective immunity induced by immunization with parasite excretory-secretory products [43]. In addition, a number of other immune mechanisms including complement activation and phagocytosis are known to be important in the control of the related hemoflagellates infecting fresh water fish, *Trypanoplasma borreli*, and *Cryptobia* species [317, 404].

This thesis is comprised of nine chapters including a review of the pertinent literature in chapter 2 where the immune responses of fish and mammals to kinetoplastid, and in particular trypanosome, infections in vertebrates are examined. Chapter 3 contains a detailed summary of the methods used to do the research. A description of the course of infection of *T. danilewskyi* in goldfish *in vivo* and the characteristics of parasite growth in culture are presented in chapter 4. Identification of antigenic components of parasite excretory-secretory products at the protein and gene levels, as well as the expression of recombinant antigen in a prokaryotic expression system, are described in chapter 5. Chapter 6 includes an examination of the effects of serum from recovered goldfish, and rabbit antibodies generated to the recombinant proteins, on parasite growth *in vitro* and the results of immunization studies using recombinant *T. danilewskyi* α- and β-tubulin in goldfish. The effects of complement on *T. danilewskyi* and the discovery of parasite resistance to complement are reported in chapter 7. Finally, the attempts to identify a putative parasite molecule involved in resistance of *T. danilewskyi* to host complement are described in chapter 8. A general discussion involving a summary of the results and recommendations for future research on this topic is presented in chapter 9.

The overall purpose of my postgraduate research was to develop a better understanding of the nature of acquired immunity to *T. danilewskyi* in fish by: (1) identifying parasite antigens that induce protective immunity in goldfish; and (2) by examining the role of antibody-mediated mechanisms of host defense that may contribute to elimination of the parasites. The specific aims of my thesis were:

1) To determine the identity of specific parasite molecule(s) found in *T. danilewskyi* excretory/secretory products that induce antibody production, and perhaps protective immunity, in goldfish.
2) To identify the genes encoding the molecule(s) identified as antigens and clone the sequences into a prokaryotic expression vector for production of recombinant proteins.
3) To determine whether the recombinant molecule(s) induce protective immunity in goldfish, and to assess the effects of parasite and antigen-specific antibodies on parasite growth *in vitro*.
4) To determine the effect of goldfish complement on *T. danilewskyi in vitro*.

CHAPTER 2

LITERATURE REVIEW[1]

2.1 Introduction:

The kinetoplastids (Phylum Euglenozoa) are a large group of flagellated protozoans containing both free-living and parasitic species. Members that are parasitic can be found infecting all groups of eukaryotes including other protozoans. The kinetoplastids are characterized by the presence of the kinetoplast, a unique organelle containing the mitochondrial DNA (referred to as the kinetoplast DNA) located in the single mitochondrion near the base of the flagellum. The kinetoplast DNA is often organized into a small number of 'maxi-circles' and a larger number of 'mini-circles'. Kinetoplastids possess other unique characteristics including RNA editing, compartmentalized glycolysis, antigenic variation, and trans-splicing [109].

There are two sub-orders distinguished within the larger group: the Bodonina possessing two flagella, and the uniflagellated Trypanosomatina. The Bodonids are primarily free-living (*Bodo*) but other representatives are ecto-or endo-parasites of fish (*Ichthyobodo, Cryptobia, Trypanoplasma*). Members of the Trypanosomatina are obligate parasites of diverse groups of organisms including ciliates, nematodes, insects, mammals, and even plants. The trypanosomatids include members of the genera *Leishmania* and *Trypanosoma*, which are intensively studied because of their medical and veterinary importance [338, 350]. Although phylogenetic analysis of the trypanosomatids has been controversial many researchers now believe that the group can be separated into two larger clusters containing the non-trypanosome trypanosomatids (*Leishmania, Leptomonas, Crithidia, Herpetomonas, Blastocrithidia, Phytomonas*) and the members of the genus *Trypanosoma*. The trypanosomes are considered to be a monophyletic group consisting of three clades: (1) the *Trypanosoma cruzi* clade that contains species originating in South American mammals; (2) the *Trypanosoma brucei* clade that contains the tse-tse fly transmitted parasites of mammals as well as the related species *T. evansi* and *T. equiperdum*; and (3) the aquatic clade that contains the leech transmitted parasites of amphibians (*T. rotatorium*) as well as marine (*T. boissoni*) and freshwater fish (*T. danilewskyi*). These relationships have been determined through the analysis of small subunit rRNA and glyceraldehyde phosphate dehydrogenase (GADPH) sequences and comparison of the surface structure of trypanosomes [117, 149-50, 282].

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In nature, *T. danilewskyi* Laveran and Mesnil, 1904 (syn. *T. carassii*) is a parasite that is found in the bloodstream of fresh water fish including carp (*Cyprinus carpio*), tench (*Tinca tinca*), and eel (*Anguilla* spp.), primarily in Europe and Asia [217]. *T. danilewskyi* is experimentally infective to a number of other species of fish including the tinfoil barb (*Barbus schwanenfeldi*), brown bullhead (*Ictalurus nebulosus*), and goldfish (*Carassius auratus*) [215, 405]. In carp it can often be found in co-infections with the related Bodonid hemoflagellate *Trypanoplasma borreli* (syn. *Cryptobia borreli*) [218]. In nature both *T. borreli* and *T. danilewskyi* are transmitted between hosts by blood feeding leeches (*Hemiclepsis* spp. and *Pisciola* spp.) [218]. While the leech is simply a vector for *T. borreli* it acts as an intermediate host for *T. danilewskyi* in which the parasite goes through a number of developmental stages in the stomach before migrating to the crop. For both species of flagellates, the infective stages are inoculated into a new host with the next blood meal [180].

Under normal circumstances *T. danilewskyi* is not considered to be pathogenic. However, in aquaculture settings, the prevalence of trypanosome infections can reach 100% [218, 283]. Mortality due to trypanosomiasis has been reported from experimentally infected fish and is normally associated with intensity of the infection. Fish infected with high numbers of parasites often succumb to infection while the mortality occurring in groups infected with smaller numbers of parasites is considerably lower [170, 389, 403]. Anemia and anorexia are symptoms that have been associated with experimental infections of goldfish with *T. danilewskyi* and histopathological changes in the hematopoietic organs have also been documented [98, 168-9]. The species of trypanosomes infecting mammals have evolved mechanisms that allow them to persist within the host and in doing so to ensure transmission to the next step of the life cycle. Members of the *T. brucei* group employ a unique mechanism to vary their predominant surface coat glycoproteins and thus avoid targeting by the immune system [49] while *T. cruzi* hides from immune surveillance within host cells. These tactics have significantly hindered progress in the development of effective vaccines against trypanosomiasis in mammals including humans. Although *T. danilewskyi* does not display antigenic variation [283] nor have any intracellular forms been identified, it is also able to establish long-term infections.

While characterization of the immune response of fish to *T. danilewskyi* is in the early stages, even less is known about the parasite and the strategies it uses to maintain chronic infections. Currently the treatment methods for hemoflagellate infections in fish are limited, and there are no commercial vaccines available. However, there are methods of disease prevention that appear promising including vector control [26, 129] and animal husbandry. In particular, strains of carp displaying genetic resistance to *T. borreli* have been identified, and additional studies have demonstrated the potential for immunization against *Cryptobia* and *T. danilewskyi* infections in fish [19, 43, 319, 406].

This review explores and compares the information that is available regarding the immune response of fish and higher vertebrates to kinetoplastid infections with emphasis on the trypanosomes, and also aims to identify some of the mechanisms that the parasites use to avoid eradication by the host defenses. The majority of the discussion of the host response to mammalian trypanosomes will involve *T. cruzi,* because fish trypanosomes have been shown to possess a similar surface coat structure, and therefore may display many of the same interactions with host defenses.

2.2 Comparison of the immune responses of mammals and fish to trypanosome infection:

The vertebrate immune system is normally discussed in terms of two branches, the innate (natural) responses and the acquired (specific) responses. Prior to the emergence of the genes encoding the hallmark components of the adaptive immune response (T- and B-cell receptors, and the MHC molecules), organisms relied exclusively on the diversity of non-specific defenses [240]. The innate immune system consists of defense mechanisms that are pre-existing within a host and are not dependent on previous exposure to a pathogen. The study of innate immunity has become increasingly popular since the discovery of homologues of many innate immune system components and signaling pathways in lower organisms, including invertebrates [160]. As a result, the innate immune system is now considered to possess striking specificity with the ability to distinguish between different types of pathogens and the host [122].

Fish represent the earliest group of vertebrates possessing the elements of both innate and acquired immunity although it is clear that their adaptive response is less developed than that of higher vertebrates [94, 391]. Despite this apparent deficiency, fish comprise the largest group of vertebrate species (~24,600), and its members can be found in the most extreme aquatic habitats. In addition, fish are in constant contact with an environment containing potential pathogenic organisms, and have evolved a number of constitutive and inducible innate immune responses to defend against infection. Conservation of many of the components of the innate response between insects and mammals suggests a common ancestry for this branch of immunity [160]. Since components of innate immunity are present in organisms lacking the elements of a specific immune system, it is believed that these pre-existing innate effector mechanisms may also play a role in the development of an effective acquired immune response. As a result, the rationale for the studies of fish immune defenses stems from an interest in deciphering the origins of the vertebrate immune system, and due to the rapid growth of the aquaculture industry, from a need to develop tools for maintaining fish health.

2.2.1 Soluble Mediators of Immunity

2.2.1.1 The Acute Phase Response

The acute phase response (APR) is an immediate inflammatory response mounted by the host in response to injury, infection, and trauma. The major roles of the response are to contain and limit the spread of infection, to prevent excessive tissue damage, and to promote healing. It is important to note that maintenance of the

APR over the long term can result in chronic inflammation and an unfavorable outcome for the host. The APR is not confined to the area of injury and involves a number of organ systems undergoing various physiological and biochemical changes including the up-regulation of the levels of plasma proteins known as the acute phase proteins (APPs). The acute phase response is mediated by cytokines including TNFα, IL-1, and most critically IL-6, secreted from stimulated macrophages, which induce the immediate production of APPs in the liver [31, 65]. The subsequent production of various chemoattractants induces migration of granulocytes and macrophages to the site of infection to initiate acute inflammation. Some of the manifestations of the acute phase response include fever, increases in vascular permeability, and enhancement of other nonspecific immune responses. While the production of a great number of plasma proteins is up-regulated during the APR, there are still others whose synthesis is actually reduced. The plasma proteins that increase by 25% or more are considered to be positive APP, and those that decrease by the same percentage are known as negative APP. Each group is further divided into minor, intermediate, and major categories according to the magnitude of change during the acute phase response. Some of the major vertebrate APPs include Serum amyloid A, C-reactive protein, and Serum amyloid P whose concentrations may rise 1000-fold [65, 371]. Many of the complement components and some of the coagulation and transporter proteins such as ferritin and haptoglobin are examples of intermediate and minor APPs respectively. Some examples of proteins whose production is down-regulated during the acute phase response include albumin and transferrin [31, 123].

Alpha-2-macroglobulin is an interesting acute phase protein that is a positive APP in some vertebrate species and a negative APP in others [31]. The down-regulation of certain proteins is in part to counteract the increased production of the positive APPs on total solute concentration in the blood. Some of the specific activities of positive APPs include leukocyte activation by serum amyloid A, resulting in the induction of chemotaxis and increased phagocytosis. They also play a role in transporting lipids to cells involved in the immune response that are undergoing proliferation and differentiation [351, 371]. C-reactive protein is an APP that seems to have both pro-inflammatory and anti-inflammatory functions. It can act as an opsonin and has the ability to activate complement by directly binding to pathogens. However, it also has a role in controlling inflammation by preventing adhesion and superoxide production by neutrophils [123]. Other mechanisms by which the acute phase response can be turned off include negative feedback control by glucocorticosteroids, as well as the increased expression of pro-inflammatory cytokine receptor antagonists that may be mediated by Th2 cytokines such as IL-4 [29]. The acute phase response to protozoan infections seems to depend on the degree of stimulation of macrophages during the infection, since these are the cells that are initiators of the inflammatory response, and a decrease in parasitemia can be correlated with the maximal concentrations of APPs [126, 324].

Trypanosome infections stimulate the production of a number of acute phase proteins, probably as a result of the parasites' ability to induce production of inflammatory cytokines including TNFα, IL-1, and IL-6 [369]. The serum protease inhibitor α-2-macroglobulin plays a role in modulating *T. cruzi* infections by binding the parasite cysteine protease, cruzipain, in both its soluble and membrane bound forms, in addition to other parasite proteases [301-2]. Trapping of cruzipain by α-2-macroglobulin results in impaired proteolytic activity of the enzyme and facilitates removal of the protease-inhibitor complexes by macrophages and hepatocytes. In fact it has been shown that receptors for α-2-macroglobulin are up-regulated during *T. cruzi* infection in mice [80]. Increased phagocytosis of the protease-inhibitor complexes can facilitate the development of acquired humoral and cell mediated responses to cruzipain through presentation to T-cells [252]. Since cruzipain has been implicated in facilitating parasite invasion of host cells, neutralization of the enzyme may result in impaired invasion of cardiomyocytes in murine models of Chagas' disease [80, 242]. A similar response was observed for pregnancy zone protein (PZP), another α-macroglobulin that has been shown to rise in concentration during some inflammatory disorders including Chagas' disease [301]. *In vivo*, increases in α-macroglobulin concentrations are associated with resistance of mice to *T. cruzi* infections and in humans, concentrations of α-2-macroglobulin have been shown to increase in children living in areas endemic for Chagas' disease with asymptomatic individuals expressing higher levels of the protease inhibitor [17, 239].

There appears to be an increase in the concentration of serum amyloid A3 in mice stimulated with *T. cruzi* GPI-anchored mucins [114]. Macrophages stimulated *in vitro* exhibit increased expression of this acute phase protein, and high levels of expression were also observed in the liver and heart tissues of *T. cruzi* infected mice. Because expression of serum amyloid A3 was associated with presence of parasites and inflammatory cells, it seems that it may be involved in the pathology of Chagas' disease in mice [114].

Little is known about the acute phase response to African trypanosomes. However, in mice infected with *T. b. brucei* the serum haptoglobin and serum amyloid P concentrations increase while the concentrations of albumin are reduced [257, 331]. Haptoglobin levels increase in response to *T. brucei* infection in strains of resistant mice and may be involved in binding hemoglobin that is released from lysed red blood cells during infection [331]. African trypanosomes are also able to up-regulate the expression of transferrin receptors that are used to scavenge iron from the host in response to low iron concentrations [107]. Thus, the parasites may take advantage of the increased concentration of transferrin during the acute phase response in some host species to obtain iron required for parasite multiplication. Furthermore, some studies [325] have reported the use of transferrin itself as a growth factor by bloodstream forms of *T. brucei*.

The heavy reliance of fish on the innate immune response, and the conservation of essential elements of the acute phase response in invertebrates has lead researchers to conclude that fish should have the capacity to mount an effective acute phase response. This would be particularly relevant under conditions such as low temperature in which the acquired immune response is impaired [31]. The studies of the production of acute phase proteins in fish have identified a number of homologues of mammalian APPs including C-reactive protein [24], serum amyloid P [224], serum amyloid A [179], and α-2-macroglobulin [347] amongst others. Many of these molecules have also been shown to change in concentration in response to inflammatory stimuli [179, 399]. Alpha-2-macroglobulin was not up- regulated following bacterial challenge, indicating that it might not be an APP in fish [32]. However, it has been proposed that the relatively high constitutive levels of this protein may play a role in innate host defense [32]. In contrast to what has been shown in most mammals, transferrin has been shown to be a positive APP in fish. This finding is understandable from two perspectives. The first is the fact that the cleavage products of transferrin have recently been found to activate fish macrophages *in vitro* to produce nitric oxide, an important component of the antimicrobial response of macrophages [346]. Second is the fact that the iron-binding properties of transferrin would act to deprive pathogens of an essential growth factor [31].

Cryptobia-tolerant fish, in which the host becomes infected but does not develop the disease, are able to neutralize the metalloprotease secreted by the parasite with the serum protease inhibitor, α-2-macroglobulin. In fact it has been proposed that the generation of transgenic fish expressing high levels of α-2-macroglobulin might represent a good strategy for protection of susceptible species of cultured fish against *Cryptobia* [404]. The increased expression of several acute phase proteins, including complement component 3 and α-2-macroglobulin, were detected in the kidney during the early phase of the infection of carp with *T. borreli*. In addition, mRNA for these two proteins and serum amyloid A were up-regulated in the liver, probably as a result of the ability of the parasites to stimulate TNFα and IL-8 receptor expression in carp phagocytes [317]. Although there has been no analysis of the pattern of acute phase proteins induced following *T. danilewskyi* infection, the inability of the parasites to stimulate an inflammatory macrophage response suggests that the acute phase response may not be important in the control of this parasite.

2.2.1.2 Trypanolytic Factors

The natural resistance of humans and some primates to the species of trypanosome infecting cattle, *T. brucei brucei*, is the result of the presence of non-immune factors in serum that cause lysis of the parasites. Alternatively, humans are highly susceptible to *T. brucei gambiense* and *T. brucei rhodesiense* because these parasites are resistant to lysis by normal human serum. The initial characterization of the trypanolytic factor found that the activity was associated with high-density lipoproteins (HDL) [310]. Further purification resulted in the identification of two trypanolytic factors, TLF-1 and TLF-2, in normal human serum. TLF-1 is a lipid rich (40%) particle containing apolipoprotein A-I, haptoglobin related protein (Hpr), paraoxanase, and apolipoprotein A-II. Initially, the proposed mechanism for TLF-1 mediated killing involved the binding of the molecule to a receptor in the flagellar pocket and subsequent endocytosis. According to this model, once the TLF is delivered to the lysosomal compartment, the low pH environment would activate peroxidase activity resulting in rupture of the lysosome and digestion of the parasite [254, 341]. However, it has since been discovered that TLF-1 is probably not active *in vivo* because its activity is completely inhibited by haptoglobin and normal serum levels of haptoglobin are high enough to prevent endogenous activity of the lytic factor [342]. Both TLFs are present in similar concentrations, share common components, and act on parasites in the same time and temperature dependent manner. The two likely differ in the way that they are bound by parasites because binding of TLF-1 is inhibited by haptoglobin. This hypothesis is supported by the observation that TLF-1 activity is high in individuals with low concentrations of serum haptoglobin [379]. TLF-2 is a much larger, lipid-poor (<1%) particle containing apolipoprotein A-I, Hpr, and multimeric IgM. TLF-2 is likely responsible for most, if not all, of the trypanolytic activity found in serum because it is not inhibited by haptoglobin. The lytic activity is severely diminished in serum that has been depleted of IgM [303-4].

Apolipoprotein A-I and haptoglobin-related protein are components of both lytic factors and are therefore likely to be required for lysis [303]. The activity of both TLF-1 and TLF-2 requires receptor-mediated endocytosis of the particles. Receptor-binding interactions have been described for a number of host proteins in the flagellar pocket of the parasites including the TLF proteins apolipoprotein A-I and Hpr [92]. As mentioned previously the mechanism by which TLFs cause lysis is not well understood; however, following receptor-mediated endocytosis it appears that both TLF-1 and TLF-2 require compartmentalization in acidic vesicles to become activated [145, 219, 305]. Although it was initially hypothesized that a peroxidative killing mechanism was involved in the destruction of intracellular vesicles and cell lysis, new evidence suggests that another unidentified pathway is probably involved. It has been shown that TLFs do not generate reactive oxygen intermediates, there is no evidence for lipid peroxidation (leading to breakdown of the lysosomal membrane), nor is their activity blocked by antioxidants [249]. Therefore, although it is generally agreed that TLFs bind to receptors in the flagellar pocket and are delivered to lysosomes, the bulk of the evidence does not support a peroxidative mechanism involved in *T. b. brucei* lysis by human serum TLFs [379].

There are a number of candidate molecules that have been proposed to be the trypanocidal component(s) of the TLFs. Initial studies focused on the ability of apolipoprotein A-I (ApoA-I) to cause parasite lysis since it is the most abundant component of HDLs. Purified native apoA-I from human serum was found to cause parasite lysis and serum lacking apoA-I had no trypanolytic activity [130]. The results of other studies have not confirmed these findings and it is now believed that the purified apoA-I may have been contaminated by the real trypanocidal factor. Testing the ability of recombinant apoA-I to mediate parasite lysis will clarify the conflicting results of these studies [379].

Haptoglobin-related protein is also a component of both TLFs and is associated with apoA-I in HDLs. The role of Hpr in TLF-mediated lysis is implicated by the ability of anti-haptoglobin (Hp) antibodies to prevent anti-parasite activity [341]. In addition it has been shown that chimpanzees have a mutation in the Hpr gene and the sera from these animals lack Hpr, TLF, and lytic activity [235]. There is evidence to contradict the involvement of Hpr in direct lysis of the parasites but it seems that Hpr may at least act as a ligand for the binding of TLFs to parasite receptors in the flagellar pocket [92].

The final candidate for the TLF toxin was recently identified through the analysis of the interactions of the trypanosome serum resistance associated protein (SRA) with TLFs. The SRA (described in more detail below) is found in some isolates of *T. b. rhodesiense* and is responsible for the resistance of these parasites to lysis by human serum. Comprehensive mutagenesis experiments designed to target the minimal essential components of the SRA involved in resistance revealed that the important residues were confined to the N-terminal α-helix that is involved in coiled-coil protein interactions. Using affinity chromatography, proteins identified as apolipoprotein L-I (apoL-I) were shown to specifically interact with the N-terminal portion of the SRA. Recombinant apoL-I was able to restore lytic activity to serum made deficient through the use of anti-apoL-I antibodies and interestingly, the gene for apoL-I was also found to be missing in chimpanzees [297, 378]. Apolipoprotein L-I and SRA have both been shown to localize to the parasite lysosomes, which correlates with what is known about the mechanism of uptake and lysis by TLFs. Still, despite the accumulation of support for apoL-I as the trypanolytic component of TLFs, the differential abilities of sera from a number of primate species deficient in apoL-I to cause parasite lysis, has not ruled out the potential contribution of haptoglobin-related protein [223].

The species of trypanosomes that are infective to humans are able to resist lysis by the natural lytic factors found in human serum. Originally, resistance was thought to occur as a result of a defect in uptake and processing of TLF-1 and TLF-2 by altered receptors or deactivation of TLF proteins [367]. The altered receptor hypothesis is supported by the studies that show that TLFs accumulate in the flagellar pocket of *T. b. rhodesiense* but that they are not endocytosed [144].

A major breakthrough in the characterization of the mechanism of resistance of *T. b. rhodesiense* to TLF occurred when a gene encoding a serum-resistance-associated protein (SRA) was identified that is expressed at high levels in resistant isolates of *T. b. rhodesiense* [78]. The SRA gene is associated with VSG switching sites and encodes a truncated VSG-like protein lacking a signal sequence. Expression of the gene is sufficient to confer resistance of transfected *T. b. brucei* to human serum in which it would normally be lysed [410]. Attempts to detect the native SRA protein have not been successful; however, antibodies to a recombinant SRA-homologue expressed in *E. coli* revealed expression of the protein on the entire surface of the parasites including the flagellum and flagellar pocket [246]. Not all strains of *T. b. rhodesiense* express the SRA gene, and, since it has not been discovered in the other species of African trypanosome infecting humans (*T. b. gambiense*), it seems that there may be more than one mechanism involved in the resistance of these trypanosomes to TLF-mediated lysis. Other candidate genes that may be involved in resistance have been identified through analysis of differentially expressed genes from serum sensitive and serum resistant strains of *T. b. rhodesiense*. An SRA-like gene has been identified from *T. b. gambiense* that is expressed in the flagellar pocket; however, stable transfection of *T. b. brucei* with the gene does not result in resistance to normal human serum [34].

The anti-parasitic effects of high-density lipoproteins (HDLs) and associated proteins from human and primate sera strongly suggest that lipoproteins and HDL particles could have evolved roles apart from their primary function of lipid transport. In particular they may be directly or indirectly involved in the innate immune defense against blood borne pathogens due to their location and high concentration in the serum. For example it has been shown that the human antimicrobial peptide hCAP-18 is bound to purified apolipoproteins and that the sequestration of these molecules is important for maintaining high levels of the protein in the serum while avoiding non-specific cytotoxic effects [344]. While the effects of apolipoproteins and other HDL- associated molecules on fish hemoflagellates have not been investigated, HDL and its component apolipoproteins A-I and A-II from carp have been shown to have antimicrobial properties. While the primary structure of the apolipoproteins is not well conserved among species, the secondary structure consisting of a high content of amphipathic α-helices is similar. Both apoA-I, apoA-II, and cationic peptides derived from apoA-I were able to inhibit the growth of bacterial pathogens and they were also able to synergize with lysozyme to enhance their antimicrobial activity [72]. Lipoproteins comprise a very large portion of fish serum compared to other vertebrate species and the presence of apolipoproteins has also been discovered in fish mucus, an important defensive barrier in these animals [71]. These results, in addition to the documented effects of apolipoproteins on mammalian trypanosomes, suggest that fish lipoproteins may play a role in the innate immune response to infection with blood-borne pathogens.

2.2.1.3 Cytokines

The processes of the immune response are under the control of a variety of soluble mediators known as cytokines. Cytokines are produced by a variety of different cell types and can act locally at the site of infection, or can be transported via the blood to affect cells throughout the body. They have a plethora of functions resulting in responses such as inflammation, cellular activation/deactivation, and local proliferation of specific cell types. In addition, the discovery of functional equivalents of cytokines in invertebrates has lead to the revelation that many vertebrate cytokines may have alternative activities mediated by lectin-like interactions in addition to their specific receptor-mediated effects [37].

Infection with a pathogen results in the production of a specific cytokine profile that directs the host to mount appropriate cellular and humoral responses to combat the invading organisms. In spite of these measures, many microbial organisms have evolved ways to take advantage of the exquisite immunological control mechanisms exerted by cytokines [73, 396]. A number of cytokines have been reported to play direct inhibitory and exacerbative roles during trypanosome infections in mammals.

Tumor necrosis factor-α (TNFα) is a pro-inflammatory cytokine that is produced by macrophages, monocytes, neutrophils, NK-cells, and T-cells. TNFα is involved in the pathogenesis of many parasitic diseases including sleeping sickness and Chagas' disease [228]. Aside from its role in the induction of anti-microbial responses in macrophages, including the production of nitric oxide (NO), TNFα exerts direct anti-trypanosome effects by binding parasite carbohydrate residues through its lectin-like domain. The lectin-like domain involved in parasite binding has been shown to be separate from the receptor binding domain involved in induction of tumor cell necrosis [222, 228]. TNFα causes lysis of *T. brucei*, *T. musculi*, and *T. cruzi in vitro* and anti-TNF treatment of *T. brucei* infected mice results in an increase in parasitemia in the blood and tissues [196, 228, 273]. In *T. brucei* the effect of TNFα is dependent on temperature and life cycle stage. Resistance of the vector stages to lysis is mirrored for TNFα treatment of *T. cruzi* epimastigotes [228, 273]. While the mechanism of action of TNFα remains unknown, the parasites have been observed to bind and take up the cytokine in the flagellar pocket. Internalization of the molecules leads to an apparent loss of osmoregulatory control and subsequent cell lysis [228-9]. It may be advantageous for parasites to use carbohydrate ligands rather than specific receptors to bind host molecules because it allows them to contend with lectin–like effectors from a number of host species. Since glycosylation patterns are not encoded in the trypanosome genetic material, many of these parasite molecules may be overlooked during the analysis of expression libraries [229]. Other mammalian cytokines possess lectin-like domains whose activities are unknown but they may play a direct role in the elimination of pathogens. One example is granulocyte-macrophage colony stimulating factor (GM-CSF), which induces the proliferation and differentiation of hematopoietic cells in the host and has also been shown to cause morphological changes in *T. cruzi* trypomastigotes [273]. GM-CSF treated parasites also display an impaired ability to infect mouse peritoneal macrophages. *T. cruzi* trypomastigotes treated with both TNFα and GM-CSF lost their infectivity, and they were lysed to a greater extent than either treatment alone. This evidence suggests that the two cytokines exert their effects in different ways, and that the morphological changes induced by GM-CSF may increase the susceptibility of the parasites to TNFα - mediated lysis [273].

Some mammalian cytokines have also been reported to act as growth factors for parasites. In the case of the trypanosomes, studies have shown that the pro-inflammatory cytokine IFNγ can stimulate the growth of a number of species of African trypanosomes. These findings remain controversial, and the role that IFNγ plays in either the control or promotion of trypanosome infection may depend on a number of factors including the species and strain of parasite, the species of model host, and the stage of the infection. An additional development has been the discovery of a trypanosome derived lymphocyte triggering factor (TLTF) that acts on $CD8^+$ T-cells to induce production of IFNγ [21, 157, 227]. Early studies demonstrated that depletion of $CD8^+$ lymphocytes results in decreased IFNγ production, suppressed growth of *T. b. brucei*, and increased survival of the rat hosts [23]. Monoclonal antibodies that react with trypanosome derived lymphocyte triggering factor prevent parasite-induced IFNγ production, and subsequent growth of the parasites [22]. TLTF is associated with the flagellar pocket, the sole area of secretion in trypanosomes, and interacts directly with the CD8 molecule on the surface of IFNγ -secreting cells to induce cytokine production [278, 375]. TLTF is produced by the parasites early in experimental infections and the host is capable of generating neutralizing antibodies that prevent the activity of TLTF during later stages of the disease [148]. Trypanosome T-lymphocyte triggering factor is the first confirmed example of a 'trypanokine', factors produced by the parasites that directly or indirectly modulate host cytokine responses. Trypanokines are a good example of a mechanism that the parasites have evolved to enable persistence in the host by mimicking normal host functions [375].

Many of the mammalian cytokines have counterparts that have been identified in fish. Despite these discoveries there remain a number of important mediators that have not yet been identified from fish regardless of the ongoing exploration of expression libraries and the sequencing of a number of fish genomes (*Fugu*-pufferfish, zebrafish-*Danio rerio*). Currently, the most well characterized fish cytokines are inflammatory cytokines such as TNFα and IL-1β, which share functional similarities with their mammalian counterparts. In addition, genes encoding type I interferons (IFNα and IFNβ) that are similar to their mammalian counterparts in sequence and gene organization have also been cloned from a number of fish species including Atlantic salmon (*Salmo salar*), channel catfish (*Ictalurus punctatus*), zebrafish, and pufferfish. Like the type I IFNs in mammals, the products of these genes are involved in regulating the immune response to viral infection [312]. Most recently, putative gene sequences encoding type II IFN (IFNγ) have been identified in the Atlantic salmon, zebrafish, and pufferfish genomes [417]. These genes display little sequence homology with their counterparts in mammals and they are not similar between fish species [312]. While no functional studies have been performed with IFNγ in fish, the presence of IFNγ inducing cytokines (IL-12 and IL-18), and IFNγ producing cells (NK and T-cells), strongly suggest that IFNγ can play a role in modulating immune responses in fish [411, 416]. In addition, IFNγ-like activity has been observed in the supernatants of mitogen-activated fish leukocytes

[135, 258]. Genes encoding cytokines involved in the down-regulation of the pro-inflammatory response, including IL-10 and TGFβ, have also been identified in fish [156, 167]. Although no functional studies have been performed with either fish IL-10 or TGFβ conservation of the motifs required for biological activity suggest that the fish homologues will act in a similar manner to the mammalian cytokines. In addition the observation of a decrease in the respiratory burst response of trout macrophages to bovine TGFβ indicates the conservation of the activity of this cytokine in vertebrates [173].

In fish the analysis of the role of cytokines in the immune response to pathogens has thus far been limited to the examination of the changes in cytokine gene expression in response to infection. For example, macrophages of carp exhibit an increase in TNFα and IL-1β expression *in vitro* and *in vivo* following exposure to *T. borreli* suggesting a role for these cytokines in the immune response against blood borne pathogens [317, 319]. Now that many of the genes encoding fish cytokines have been identified and sequenced, it will be possible to produce recombinant molecules and develop antibodies in order to more directly determine the role of these important mediators in regulating the fish immune response to infection.

2.2.1.4 Antimicrobial Peptides

Antimicrobial peptides are small (12-50 amino acids) molecules that have a broad spectrum of activity against bacterial, fungal, viral, and protozoan pathogens [151]. The localization of antimicrobial peptides to host epithelial cells and mucosal surfaces is a testament to their important role in the first line of defense against invading pathogens [47, 414]. In addition to their direct microbicidal effects, antimicrobial peptides have been shown to have other roles in inflammatory responses, including recruitment of neutrophils and fibroblasts, promotion of mast cell degranulation, enhancement of phagocytosis, and decreasing fibrinolysis. In order to prevent tissue injury associated with chronic inflammatory responses, antimicrobial peptides stimulate apoptosis of activated or infected cells, decrease cytokine production, and neutralize bacterial lipopolysaccharide (LPS) [151, 186, 413-4].

To date, it has been estimated that greater than 1,000 antimicrobial peptides have been described and characterized. The properties of these molecules that are important for their biological activity include: (1) a net positive charge resulting from an abundance of basic amino acids; and (2) the ability to form amphipathic structures that allow them to interact with the main target of activity, the microbial plasma membrane. Some molecules are specifically active against pathogens, while others have also been shown to be cytotoxic to host cells [55]. The ability of antimicrobial peptides to interact with the membrane is dependent on the interaction of the positively charged peptide with the overall negative surface charge of the pathogen [151]. It has been shown that the outer leaflet of the microbial membrane contains a large proportion of negatively charged phospholipids (phosphatidyldyglycerol, sphingomyelin). In addition the microbial membrane may be decorated by other negatively charged molecules such as lipopolysaccharide (LPS) on the surface of Gram-negative bacteria. In contrast, the surfaces of most host cells possess a neutral charge distribution, and this difference seems to account for the specificity of the peptides for microbial membranes [186, 414]. Many studies [151] have shown that the activity of antimicrobial peptides can be inhibited by the presence of cholesterol in the membranes and high concentrations of salts, serum, and proteases in the environment. Although the precise means of killing by cationic peptides is not completely understood for many of the described molecules the majority seem to interfere with the stability of the plasma membrane by inducing fatal depolarization through the creation of holes/pores in the membrane leading to leakage of cellular contents and eventual cell lysis. In addition to having a direct microbicidal effect as a component of the innate immune response, the activity of antimicrobial peptides has been identified as one of the ways in which the innate immune system can regulate the activity of the acquired branch of immunity. Their role in regulation of the acquired immune response includes the attraction of monocytes, immature dendritic cells, and T-cells, as well as the enhancement of chemokine and IgG production [288].

Compared to other classes of vertebrates, the quantity and diversity of mature antimicrobial peptides identified in fish is relatively small. However many species of fish possess a number of other defense molecules that can be found at mucosal and epithelial surfaces that are also important in innate defense. These molecules include natural antibodies, apolipoproteins, lysozyme (of which a number of different isotypes have been identified), non-peptide antimicrobial compounds such as squalamine (a 655Da cationic steroidal compound from the dogfish shark), and most recently cationic steroidal derivatives from catfish peripheral blood leukocytes [25, 71-2, 110, 251, 281]. The peptides that have been identified in fish have been isolated from epithelial cells and mucous layers of the gills, skin, and intestine. Only a small number of molecules have been isolated from blood cells. Fish antimicrobial peptides have broad-spectrum activity at sub-micromolar concentrations against a wide range of human and fish pathogens, including bacteria, fungi, viruses, and protozoa. In the bony fish (teleosts), a growing number of cationic peptides have been isolated from a variety of species. Most of these molecules have been isolated from the epidermal cells or secretions of the skin, gills, and intestine. Many of the fish antimicrobial peptides have high sequence homology to segments of other proteins (particularly histone or histone-like molecules) indicating that they may be cleavage products of larger molecules. Cleavage peptides have been isolated from the channel catfish, rainbow trout, hybrid-striped bass (*Morone* spp.), as well as Coho (*Oncorhynchus kisutch*) and Atlantic salmon [261, 313]. A number of specific antimicrobial molecules characterized in rainbow trout (*Oncorhynchus mykiss*), called onchorhyncins, have been found to be very similar to chromosomal proteins [110-12]. The remaining antimicrobial peptides isolated from fish are a heterogeneous group of compounds; the majority of which are known to form amphipathic α-helices. They include: the pardaxins [279], misgurin [286], the pleurocidins [69], the piscidins and moronocidins [204, 337], the chrysophsins [166], and two hydrophobic, pore-forming peptides from carp [99].

Very few peptides from fish show hemolytic activity, and there are examples of peptides that retain their activity in high salt concentration environments. Small, cationic antimicrobial peptides are important components of the non-specific defenses of many forms of life, including fish. Their vital role in establishing immunity in fish is demonstrated by: (1) their localization to the primary sites of infection (gills, skin, gastrointestinal tract); (2) their up-regulation following infection or injury; and (3) their demonstrated ability to protect salmon from bacterial infection *in vivo* with continuous injection of peptide.

There have been a limited number of studies performed to determine the effects of antimicrobial peptides on trypanosomes; however, peptides having anti-parasite activity towards *Leishmania* and *Plasmodium* have been identified [386]. The observation that apolipoprotein A, which has been shown to have characteristics that are similar to antimicrobial peptides, can cause lysis of trypanosomes suggests that other small cationic peptides may have anti-trypanosome activity. For example, cecropin, a linear cationic peptide originally isolated from the giant silk moth *Hyalophora cecropia*, has anti-*T. cruzi* activity. Trypomastigotes of *T. cruzi* are killed by synthetic cecropin-like peptides *in vitro* [27]. The same researchers witnessed a decrease in the number of amastigotes per cell in a *T. cruzi*-infected cell line treated with the peptides. *In vivo*, *T. cruzi* infected mice that were injected every day for 10 days with cecropin showed reduced parasitemia and mortality [27].

Some antimicrobial peptides are non-specific and cause lysis of the host cells as well as the pathogen. As a result, these molecules do not represent good chemotherapeutic agents for the treatment of disease. Dermaseptins (present in the skin secretions of amphibians) have been shown to have rapid anti-*T. cruzi* effects; however, they do not harm human leukocytes [52].

The finding that *T. cruzi* is resistant to perforin, an effector molecule used by $CD8^+$ T lymphocytes and NK cells to lyse target cells, suggests that there are other molecules that may act instead of, or in synergy with, perforin to facilitate the protective NK/ $CD8^+$ response. NK-lysin, and the human homolog granulysin, are produced by cytotoxic T-cells and NK cells and are effective against a variety of microorganisms *in vitro*. NK-lysin and a shorter synthetic peptide NK-2, which was engineered to eliminate its ability to lyse host cells, have been shown to destroy *T. cruzi in vitro*. Exposure of the parasites to NK-lysin or NK-2 results in the permeabilization of the cell membrane as a result of trans-membrane pore formation leading to cell lysis [171].

Trypanosoma brucei has also been shown to be susceptible to a variety of antimicrobial peptides, and the results of these studies have generated interest in the molecules for therapeutic applications, and as candidates for transgenic expression in bacterial symbionts of the parasite vector. Members of the cathelicidin family of peptides appear to be the most effective at lysing both the vector and bloodstream forms of the parasites via a mechanism that involves the disruption of surface membrane integrity [237]. In one experiment, the peptides that were found to be effective at killing the trypanosomes had no effect on a bacterial symbiont found in the midgut of the tsetse fly, *Sodalis glossinidus*. These molecules have been identified as excellent candidates for transgenic expression in *S. glossinidus*. It is hoped that expression of the peptides will prevent the vectors from maintaining and transmitting the parasites [146].

The role of naturally occurring microbicidal molecules in the immune response of fish to protozoan parasites has only been studied for *Amyloodinium ocellatum* infections in rainbow trout and hybrid striped bass. *Amyloodinium ocellatum* is an ectoparasitic dinoflagellate that can cause serious disease in marine aquaculture. Antimicrobial proteins were isolated from skin, gill, and spleen and the isolated peptides were able to inhibit the trophont (feeding stage of the parasite) while the dinospore (stage involved in transmission) remained unaffected. The isolated peptides were closely related to histone H2B and H1, and treatment of the parasites with the purified histone-like molecules resulted in reduced growth, differentiation, and infectivity [261]. Additional studies revealed that the histone-like peptides could be localized to the fish epidermis although the mucus also contained some anti-parasite activity. Interestingly, the histone-like antimicrobial peptides had the ability to directly kill the parasites, induce developmental abnormalities, and cause delayed mortality [262].

An increasing number and variety of antimicrobial molecules are being identified from fish. Since many cationic peptides from other vertebrates have been shown to have trypanocidal activity the effects of these natural antibiotics, in addition to those discovered in fish, on *T. danilewskyi* could be examined to determine their role in the innate immune response.

2.2.1.5 Complement

The complement system is an evolutionarily ancient component of the innate immune system that plays a role in tagging target cells for killing and immune complexes for removal. In mammals the complement system consists of more than 30 serum and membrane associated proteins that function in activation and regulation of the cascade [388]. The majority of complement components have characteristic domain structures that have allowed their evolutionary history to be traced [265]. Central components such as complement component three (C3) and serine proteases involved in initiating the complement cascade have been discovered in sea urchins (the earliest deuterostomes) as well as the ascidians (deuterostome invertebrates [264]. No complement component homologues have been discovered amongst the protostomes nor have any gene sequences been identified from various protostome genome projects. These results suggest that the complement system is in fact an innovation of the deuterostome lineage. Thus, the evolution of the organized complement system as it is found in mammals pre-dates the appearance of acquired immunity and represents one of the oldest and perhaps most organized mechanisms of innate immunity.

Activation of complement occurs in a sequential manner via three different pathways that result in a variety of biological activities including: (1) opsonization of targets; (2) chemotaxis and activation of leukocytes; (3) direct killing/cytolysis of targets; and (4) enhancement and regulation of adaptive immunity. The three pathways of complement activation include the alternative, the lectin, and the classical pathway. All three pathways are triggered via different mechanisms; however, all three converge at the proteolytic activation of the central molecule complement component three (C3) [388]. The alternative pathway does not require specific recognition molecules and results in the tagging of activating surfaces with C3 bound to hydroxyl and amine groups. Subsequent attachment of factor B leads to amplification of C3 cleavage and C3b binding in the absence of complement regulatory proteins [206]. The lectin pathway involves recognition of specific pathogen-associated carbohydrates such as mannose by mannose-binding lectin (MBL) and *N*-acetylglucosamine by ficolins. MBL and ficolins are associated with

MBL-associated serine proteases (MASPs). These enzymes can cleave C3 directly, or participate in forming the C3 convertase enzyme by cleaving C4 and C2 [121]. Cleavage products of C4 (C4b) and C2 (C2a) form the C3 convertase enzyme of the lectin and classical complement pathways leading to deposition of C3b on target surfaces. The classical pathway is initiated by the binding of the recognition molecule C1q to the Fc portion of antibody-antigen complexes resulting in the activation of the associated serine proteases C1r and C1s that cleave C4 and C2 in a manner similar to that described for the lectin pathway [206]. The classical pathway is found only in jawed vertebrates and is believed to have evolved coincident with the appearance of adaptive immunity.

Cleavage of C3 and deposition of C3b on the surface of targets has two potential outcomes. The first is initiation of the formation of the membrane attack complex (MAC) that results in target cell lysis. In addition, opsonization of the targets with C3b leads to increased uptake by phagocytic cells through the binding of C3 receptors on the surface of the cells. In mammals, C3b binds primarily to complement receptor 1 (CR1). Many of the phagocytic cells are also antigen-presenting cells (APCs) and therefore complement can facilitate the antibody response to T-cell dependent antigens by enhancing phagocytosis, antigen processing, and antigen presentation to T-cells. Phagocytic cells also possess receptors for breakdown products of C3b that are known to participate in a variety of immune responses [125]. The smaller products of the enzymatic cleavage of C3/4/5 are the anaphylatoxins and these small peptides have an evolutionarily conserved role in the regulation of inflammation through binding of specific receptors found on the surfaces of leukocytes [66, 206, 352]. The anaphylatoxins are chemoattractants known to act locally to induce degranulation and respiratory burst as well as to stimulate phagocytosis and cytokine release in inflammatory cells. In mammals, the anaphylatoxins have also been shown to have systemic effects including increased vascular permeability and smooth muscle contraction.

Since C3 is continually susceptible to activation, and since it may be deposited on the surface of non-target cells, excessive complement activation is prevented by the activity of complement regulatory proteins such as factor I and factor H [206]. In the presence of soluble and membrane-bound co-factors such as factor H, C4 binding protein, and membrane co-factor protein, factor I has the ability to regulate complement activation by cleaving C3b and C4b into C4c and iC3b. iC3b cannot participate in formation of the C3 convertase but it can further be degraded into C3dg and C3d [206]. In mammals, iC3b is an important opsonin that enhances phagocytosis of coated targets by leukocytes bearing complement receptors 3 and 4 (CR3 and CR4) [125]. C3d-antigen complexes have been shown to induce and enhance antibody responses by binding to complement receptor 2 (CR2) on the surface of B-cells while simultaneously binding the B-cell receptor. The threshold for B-cell activation is decreased significantly in the presence of antigen, and for this reason it is believed that C3d acts as a natural adjuvant *in vivo*, and provides a clear link between innate and adaptive immunity [82].

The complement system of the teleost fish is the most intensively studied among the lower vertebrates. Functional studies as well as the isolation and/or cloning of genes for most of the complement components have provided strong support for the existence of all three pathways of activation, as well as a functional lytic pathway. The most intriguing difference between the teleost complement system and the complement systems of other vertebrates is the structural and functional diversity of some of its components. Teleosts possess a large repertoire of genes encoding the complement components, and some of these genes also demonstrate allelic polymorphism. The presence of multiple copies of some of the central complement proteins, such as C3 and factor B, has generated a great deal of interest in determining the functional purpose of these duplications [352].

Multiple genes encoding structurally and functionally different C3 molecules have been identified in rainbow trout, carp, gilthead sea bream (*Sparus auratus*), medaka (*Oryzias latipes*), and zebrafish suggesting that diversity in C3 is a unique feature common in the teleost lineage. Some of the earliest studies involved the isolation of four different C3 proteins from the serum of rainbow trout [263, 353]. The rainbow trout C3 isoforms were found to bind differentially to various complement-activating surfaces (zymosan, sheep and rabbit erythrocytes), suggesting that the evolution of multiple C3 molecules may aid in the recognition of a variety of pathogenic organisms [353]. Eight cDNA clones encoding five different C3 isoforms have been discovered in carp, and like the rainbow trout proteins, they differ in the primary structure of important functional sites that likely affect their substrate specificity [255]. The differences in the binding specificities of these molecules is believed to play a role in expanding the recognition capabilities of the innate immune system in teleosts, which may help to augment their relatively poor antigen-specific antibody responses.

The only complement component for which functional variants have been identified in humans is C4 A/B [33]. Although C4 A and B have similar sequences there is variation in the reactivity of the thioester site due to an amino acid substitution of the catalytic histidine residue for aspartic acid in C4 A. This slight change in primary sequence has been shown to be responsible for the surface substrate specificity of C4A for amino groups and C4B for hydroxyl groups [329].

One of the most interesting implications for the existence of functionally distinct isoforms of many of the complement components in teleosts, particularly the C3/4/5 molecules, is the possibility that the cleavage products of these molecules may possess their own diverse functions. The C3a, C4a, and C5a molecules are conserved in teleost fish, and studies have shown that activation of serum using LPS or zymosan, can induce chemotaxis, respiratory burst, and phagocytosis [50, 190]. Interestingly, it has recently been shown that mammalian C3a has antimicrobial properties [13]. Accordingly, it would be interesting to determine whether the C3a molecules generated from the cleavage of the different C3 isoforms of teleost fish also possess microbicidal activity, perhaps with differing substrate specificities.

Much of the literature regarding the role of complement in immunity to trypanosome infections is dedicated to the mechanisms that these blood-borne pathogens use to resist complement-mediated lysis. Resistance mechanisms are most effective during the acute phase of the infection before the generation of specific antibodies. It is not surprising that blood-borne parasites are resistant to complement because they are exposed to the selective pressure of being in direct contact with humoral and cellular immune components found in blood. The adaptations trypanosomes have made to exist in the vertebrate host are particularly evident when compared to the susceptibility that has been demonstrated for the corresponding vector-borne stages. Still, for many mammalian trypanosome infections, and for *T. borreli* and *C. salmositica* infections in fish, the eventual generation of specific antibodies creates an opportunity for complement to play a role in either direct lysis of the parasites, or as an opsonin that potentiates the uptake of the parasites by phagocytic cells [253, 317, 358, 404].

The trypomastigote and metacyclic trypomastigote stages of *T. cruzi* are resistant to the effects of the alternative complement pathway while the epimastigote stage from the insect are highly susceptible [316, 373]. The bloodstream trypomastigotes are known to possess a number of molecules that either directly, or indirectly, inhibit the activation of complement. Most recently it has been discovered that the *T. cruzi* homologue to mammalian calreticulin, an evolutionarily conserved calcium binding protein, is associated with the ability of the parasites to modulate the host classical pathway of complement activation. Parasite calreticulin has been shown to bind C1q, the pathogen recognition component of the classical complement pathway, and thus prevent formation of the C3 convertase and subsequent downstream events [116]. The finding that parasite calreticulin blocks complement activation at the earliest stage of the classical complement pathway contributes to our understanding of the resistance of *T. cruzi* trypomastigotes to lysis. An important side effect of the impaired ability of the host to activate complement is the build-up of immune complexes that may initiate inflammatory responses and contribute to pathogenesis [115-6]. In addition, the parasites also possess a 160 kDa glycoprotein expressed on their surface that has significant homology to the host complement regulatory molecule decay-accelerating factor (DAF) [268]. The DAF-like molecule is known as the complement regulatory protein (CRP) and it exerts its activity by binding to C3b and C4b to prevent C3 convertase formation. The importance of this molecule for protection of the parasites from complement has been demonstrated by its ability to protect normally susceptible epimastigotes from lysis when they are transfected with the gene for CRP [267].

T. cruzi possesses an additional molecule that indirectly plays a role in preventing complement-mediated parasite lysis. The *trans*-sialidase enzyme is a unique form of the sialidase enzymes, which are utilized throughout the animal kingdom to adorn glycoproteins with sialic acid residues. *T. cruzi trans*-sialidase is involved in mediating the transfer of sialic acid residues from host glycoproteins to the GPI-anchored, carbohydrate-rich, mucin-like proteins that coat the surface of the trypanosomes [70]. The maintenance of a sialic acid-rich glycoprotein coat confers an overall negative charge to the parasite surface that is thought to inhibit binding of complement components and interestingly, is a mechanism in which the host prevents non-specific complement-mediated lysis of its own cells [366]. In addition to its role in protecting the parasites from complement attack, *T. cruzi trans*-sialidase has been implicated in the invasion of host cells [326], and contributes to immunosuppression [124]. Despite the evasion strategies of the trypanosomes, specific antibodies are eventually generated to the parasites. Many of the antibodies recognize molecules involved in the resistance to complement and render the parasites susceptible to lysis by activation of the alternative complement cascade [199]. Immunization of mice using some of the targets of lytic antibodies, including CRP and *trans*-sialidase, has been shown to confer protection against re-infection [269, 330, 380]. Pre-existing or natural antibodies cross-reacting with parasite surface epitopes such as anti-α-galactosyl antibodies, which are enhanced as a result of chronic parasite infection, have also been shown to cause complement-mediated lysis of trypanosomes [12].

Most of the parasites in an African trypanosome infection are cleared by liver macrophages in an antibody-dependent fashion that may be augmented by opsonization of the parasites with complement components. However, due to the mechanisms of antigenic variation, trypomastigotes that do not express the dominant variable surface glycoprotein will not be susceptible to antibody-mediated phagocytosis and will survive to establish the subsequent wave of parasitemia [44, 138]. A molecule on the surface of trypanosomes that is similar to gp63 (the surface protease of *Leishmania*) has been implicated in the resistance of the parasites to complement mediated lysis [100]. The trypanosomes possess multiple, non-identical copies of the gp63 gene. While the genes are transcribed at an equal level in both vector and bloodstream stages of the parasite, the mRNA accumulates to a far greater extent in the trypomastigotes and the procyclic forms are known to be highly susceptible to complement. Although a direct role for the gp63-like molecules in complement evasion has not yet been demonstrated for African trypanosomes it is one of the known roles for the protein in *Leishmania* [88].

Although complement activity does not seem to play a role in either of the rodent trypanosome infections, innate resistance of mice to infection with *T. lewisi* involves the activity of complement and phagocytic cells. It was also shown that rat serum proteins are involved in coating *T. lewisi* to make the parasites less susceptible to lysis by mouse serum. This strategy may delay the exposure of underlying molecules that will activate mouse complement. It is known that *T. lewisi* deprived of their surface coats are susceptible to direct complement-mediated lysis by sera from a variety of species. In the mouse, *T. lewisi* antibody-antigen complexes stimulate activation of the complement system while in *T. musculi* infections of the same host there is little to no complement activation observed [83].

Like the immune response of mammals to trypanosome infection, carp antibodies capable of lysing *T. borreli* are not produced until later in the infection when they have been shown to cause parasite lysis via activation of the classical complement cascade [317]. In contrast, *T. danilewskyi* is not susceptible to complement-mediated lysis regardless of the presence of specific antibodies. Resistant parasites can be rendered susceptible to lysis by the alternative complement pathway after removal of surface molecules by trypsin. Trypsin-treated parasites are able to regain resistance to lysis, and the restoration of resistance is dependent on the ability of the parasites to synthesize protein [296]. As mentioned previously, the surface coat of *T. danilewskyi* is similar to that of *T. cruzi* [3, 214]. In contrast to what was observed following removal of sialic acid from *T. cruzi* surface molecules, treatment of *T. danilewskyi* with sialidase enzymes did not increase the susceptibility of the parasites to complement [296]. It is possible that the fish trypanosomes may possess other molecules that restrict complement activity but these are yet to be characterized. Differential complement activity is associated with susceptibility and resistance of brook charr (*Salvelinus fontinalis*) to *Cryptobia* infection. Fish that are innately resistant to infection with *C. salmositica* prevent the infection by lysing the parasites through the alternative complement pathway. This trait is heritable in brook charr and thus can be used to select for populations of fish genetically resistant to infection [404].

2.2.1.6 Antibody responses

2.2.1.6.1 Natural antibodies

Polyreactive antibodies (including auto-reactive antibodies) have been detected in the sera of healthy individuals, including lower vertebrates, and are particularly common early in life. In mammals, they are primarily of the IgM isotype, but may also be of the IgG isotype, and they bind conserved epitopes from a variety of species. They are associated with antimicrobial defenses, particularly in the lower vertebrates. Natural antibodies differ from the monospecific pathogen-induced antibodies in that the former can be encoded in the germline with little somatic mutation while the latter are the product of rearranged gene segments. In terms of function, it seems that polyreactive natural antibodies may be required for rapid protection while the induced monospecific antibodies are more important for recovery and the generation of memory [51]. In particular, naturally occurring antibodies can lyse cells in the presence of complement and can contribute to the opsonization of target cells in the early stages of an infection [197]. Fish are not able to mount strong specific antibody responses. Thus, the humoral responses of the lower vertebrates may be controlled in part by polyreactive antibodies [51, 133].

Expression and production of mitogens and superantigens is a common evasion strategy used by parasites to induce polyclonal lymphocyte responses, particularly in the acute phase of the infection, that allow pathogens to establish in the host. Evidence for polyclonal T- and B-cell activation during the acute and chronic stages of an infection includes the development of splenomegaly, immunosuppression, hypergammaglobulinaemia, uncontrolled inflammation including toxic shock syndrome, and the promotion of self-reactive clones leading to autoimmune responses. Production of natural antibodies and low-affinity polyreactive IgM has been primarily associated with CD5$^+$ B-cells (B-1 cells in humans and mice) [58, 64]. Toll-like receptor activation may also be associated with the polyclonal lymphocyte activation that is observed during parasite infections and the generation of self-reactive antibodies. The proposed mechanism for the production of these antibodies is through the simultaneous activities of antigen binding and toll-mediated signals to B-cells [236]. In some cases the antibodies that are produced cross-react with parasite antigens. For example, some β-galactosidase-binding antibodies also recognize parasite antigens. Moreover, natural antibodies involved in autoimmunity may be important in the innate immune response to parasitic infections [197].

It has been proposed that some natural antibodies are derived from specific antibodies generated to commensal gastrointestinal bacteria. In particular, anti-α-galactosyl antibodies are found in healthy humans throughout their lives. These antibodies may be generated through constant stimulation by gastrointestinal bacteria expressing Gal (α1-3) Gal residues on lipopolysaccharide [363]. Natural antibodies specific for a carbohydrate epitope containing α-galactosyl residues (also found on the cell wall of gastrointestinal bacteria) are common in *T. cruzi* infected individuals. Direct or complement mediated lysis, of bloodstream or metacyclic trypanosomes respectively, can result from binding of anti-Gal (α1-3) Gal antibodies [12, 128].

Resistance to *T. cruzi* infection has been shown to be associated with reduction of polyclonal lymphocyte activation [323]. Hypergammaglobulinaemia appears early in the infection and is characterized by the production of large quantities of IgA, IgM, and IgG. The highest concentrations of autoantibodies were found to be of the IgM and IgG classes, and some are cross-reactive with parasite antigens. Some of the polyreactive antibodies that are stimulated by *T. cruzi* infection are generated toward negatively charged epitopes including tubulin. Antibodies that recognize these molecules are also present in pre-infection serum; however, the binding kinetics of anti-tubulin antibodies are different for serum isolated from infected and uninfected animals [185]. It has been hypothesized that these immunological phenomena may be induced by the parasite [136].

A specific parasite protein (TcPA45) was isolated from *T. cruzi* culture and was able to stimulate B-cell proliferation. The gene for the molecule was found in different stages of the parasite, and it has been postulated that it is secreted or expressed intracellularly. The protein was identified as the first eukaryotic amino acid racemase that is capable of catalyzing the conversion between L- and D-proline enantiomers [192]. A recombinant protein was produced that could induce B-cell proliferation and racemise both L- and D-proline. The maintenance of the enzyme active site was shown to be necessary for both activities. Immunization with DNA encoding TcPA45 was attempted to prevent the induction of non-specific B-cell proliferation upon *T. cruzi* infection. Control of parasitemia was enhanced significantly, particularly when sub-mitogenic doses of the protein were injected [247].

Cattle produce IgM antibodies that react with non-parasite antigens following *T. congolense* infection. Because the presence of these antibodies was also detected in pre-infection sera, it was determined that there was a pre-existing pool of antibodies that was further enhanced after infection [57]. The IgG fraction from the same animals bound specifically to parasite-derived molecules and was found only in post-infection sera, suggesting the induction of IgG by the parasite. These researchers suggested that the non-specific antibodies generated during trypanosome infection were polyreactive IgM molecules, and since the antibodies are found in pre and post-infection sera, do not represent non-specific polyclonal activation because the presence of specific antigen was not required for their development. The precise function of the pre-existing IgM in the control of parasite infection, if any, has not been fully elucidated [57].

Due to the lack of class-switching and affinity maturation of induced immunoglobulin molecules in fish, the pool of natural antibodies may be more important for host defense against invading pathogens [340]. *T. borreli* stimulated both parasite specific and non-specific antibody production in carp [317]. Alternatively, infection may amplify a pool of natural antibodies/B-cells already present in the fish. Stimulation of carp lymphocytes was not directly mediated by the parasite. In fact it has been shown previously that infection with high numbers of parasites inhibited proliferation of peripheral blood leukocytes [182]. The results of additional studies have suggested that the specificity of the antibody response might be dependent on the size of the initial inoculum. Specifically, injection with large numbers of parasites resulted in increased mortality, perhaps due to the stimulation of a polyclonal lymphocyte response, while infection with low numbers of parasites resulted in a more specific antibody response [317].

2.2.1.6.2 Specific antibodies

There are five classes of antibodies in mammals IgG, IgA, IgE, IgM, and IgD. Each immunoglobulin molecule is composed of two heavy chains and two light chains and although most exist primarily as monomeric molecules, some can be found as dimers (IgA) or pentamers (IgM) [276]. Antibodies perform a variety of functions in the immune response to parasitic infections, including the neutralization of soluble and cellular antigens, and the enhancement/activation of other immune mechanisms such as phagocytosis, antibody dependent cellular cytotoxicity, and the complement cascade. One important benefit of the mammalian specific antibody response is the generation of immunological memory. This anamnestic response involves the production of higher antibody titers having greater affinity for the microbial antigens upon secondary exposure to the infectious agent. Not surprisingly, antibody-mediated immune responses are most effective against extracellular pathogens.

It is well known that fish antibodies are of lower affinity and diversity than those of the higher vertebrates [93]. There have been a number of proposed explanations for these differences including variations in (1) gene structure (arrangement and orientation of V-D-J and constant domain segments); (2) mechanisms of generating diversity (gene conversion, somatic hypermutation); and (3) in the diversity of antibody classes. Most species of teleost fish possess only IgM antibodies that are not pentameric as in mammals, but tetrameric. The tetrameric antibody may not be as efficient at cross-linking antigen on the target surface. In addition, effective binding of IgM requires the presence of epitopes that are repeated on a single organism [294]. Some species of fish also seem to possess monomeric and dimeric forms of IgM, but their functional significance is unknown [397]. Another difference in the antibody response of fish compared to mammals includes the temperature dependency of the response [368, 397]. Interestingly, fish seem to lack a true anamnestic response to a secondary antigen exposure but this opinion is controversial [184, 397].

Antibody responses of the higher vertebrates are important in the control of trypanosome infections, particularly as the disease enters the chronic phase, because the parasites have been shown to induce transient immunosuppression during the early acute phase of the infection. For the mammalian trypanosomes *T. cruzi*, and *T. brucei*, the generation and effectiveness of the antibody response is dependent on other accessory components of both the innate and acquired immune responses, described in greater detail elsewhere in this review. Also, the components of the parasite surface coat, and the ability of both trypanosome species to evade host defenses undoubtedly determines the efficacy of the specific humoral immune response.

The surface coats of *T. cruzi* and *T. brucei* are very different in terms of their surface composition. The surface of *T. cruzi* is composed of GPI-anchored mucin-like glycoproteins encoded by a variable gene family that are not very antigenic. These mucin-like molecules have additional roles in the down-regulation of the host immune response [2, 56]. Since *T. cruzi* has an intracellular stage, cytotoxic cells have a greater role than specific antibodies in limiting parasite loads in infected hosts. However, specific antibodies generated towards trypomastigote surface proteins, as well as secreted antigens, are involved in activation of the complement cascade leading to elimination of the bloodstream forms during the acute and chronic phases of infection [12, 330, 372].

Alternatively, the surface structure of the members of the *T. brucei* group is composed of a blanket of proteinaceous, GPI-anchored, variable surface glycoproteins (VSGs). There are more than 1,000 genes encoding different VSGs in the parasite genome. However, the majority of parasites in a population only express one VSG at a time. Since the VSGs are highly immunogenic, the host is able to mount an effective antibody response to the dominantly expressed VSG, resulting in the elimination of the extracellular parasites from the bloodstream, mainly via antibody-dependent phagocytosis [138]. A small proportion of the population expressing an alternate VSG, or a mosaic of VSGs, will not be recognized by antibodies that recognize the dominant VSG and will undergo proliferation to form the next wave of parasitemia. Therefore, resistance to *T. brucei* spp. is dependent on the production of a specific antibody response to the VSG expressed by the majority of the trypomastigotes in the blood. However, VSG switching by the African trypanosomes, in addition to the immunosuppression characteristic of trypanosome infections, results in the establishment of chronic infections in mammals [44, 59].

Fish mount specific antibody responses to the kinetoplastid parasites *T. borreli* and *C. salmositica* that have been linked to protection from infection. For *T. borreli* infections in carp, antibodies are produced after a period of lymphocyte unresponsiveness, similar to that observed for mammalian trypanosome infections. The antibodies bind to the parasites and cause lysis by activation of the classical complement pathway [317]. Antibodies that are generated towards *C. salmositica*, also fix complement and augment phagocytosis of the parasites by macrophages [404]. The immune response to *T. danilewskyi* is also thought to be antibody-mediated, because recovered fish are refractory to re-infection, and passive transfer of serum or purified IgM from recovered fish can confer protection to naïve hosts [283, 390, 403]. Despite the results of these early studies, very little is known about the mechanism of antibody-mediated immunity to *T. danilewskyi*. For example, immunization of goldfish with *T. danilewskyi* excretory/secretory products, in conjunction with Freund's complete adjuvant, can protect naïve hosts from a homologous challenge infection. However, a definitive role for antibodies in mediating immunity of vaccinated fish has not been confirmed [43].

2.2.2 Cellular Mediators of Immunity

2.2.2.1 Innate immune response

Elements of the innate immune system can be non-specific, and are rapidly mobilized to prevent establishment and subsequent dissemination of infection. These natural defenses consist of physical and chemical barriers, phagocytic and cytotoxic cells, and soluble effector molecules. Innate immune responses are the first line of defense against trypanosome infections and are important for limiting replication of the parasites while the acquired immune response is being generated.

2.2.2.1.1 Pattern recognition receptors (PRRs)

The ability of the sentinel cells of the innate immune system, macrophages and dendritic cells, to respond to a variety of intracellular and extracellular pathogens is mediated by receptors (pattern recognition receptors-PRRs) that are able to recognize specific microbial signatures (pathogen associated molecular patterns-PAMPs). The pattern recognition receptors are genome-encoded molecules with predetermined specificity for PAMPs that are shared by particular classes of pathogens (*e.g.* lipopolysaccharide (LPS) of gram negative bacteria), and they tend to be highly conserved among different organisms. To combat the variety of pathogenic organisms in the environment, hosts have armed themselves with a number of different soluble, cell surface, and intracellular PRRs including complement proteins (mannose binding lectin), CD14, toll-like receptors (TLRs), and scavenger receptors. Engagement of the various PRRs can activate important humoral and cell-mediated effector systems including the complement cascade, and phagocytosis. In addition, activation of the cells of the innate immune system by PAMPs results in the production of co-stimulatory molecules and chemoattractants that recruit other cell types (including T-cells) to the site of infection, that are essential for the initiation of the acquired immune response [236].

In most cases, activation of the effector cells of the innate immune system is sufficient to control an infection. However, when the innate immune response fails or is subverted, it instructs the adaptive immune system about the nature of the infection through the up-regulation of co-stimulatory molecules on antigen presenting cells (APCs), such as dendritic cells (DCs). One particular class of PRRs that provide an important link between the innate and acquired immune systems is the toll-like receptors (TLRs). Activation through any of the ten different mammalian TLRs, present on the surface of immature DCs residing in the tissues can cause the cells to undergo maturation. Mature DCs up-regulate co-stimulatory molecules, MHC expression, and antigen processing. The mature DCs migrate to the lymph nodes where they present antigens to naive T-cells to activate the appropriate acquired immune defenses [39, 172].

The original toll receptor was identified in *Drosophila* and is involved in larval development. Later, it was also identified as part of the fly defense system against fungal and bacterial pathogens [210]. Toll-like receptors have subsequently been discovered in a number of other organisms including fish. The first teleost TLR was characterized in our laboratory from a stimulated goldfish macrophage cDNA library enriched for differentially expressed genes by suppressive subtractive hybridization [345]. Shortly thereafter, TLR genes were identified in pufferfish, zebrafish, Japanese flounder (*Paralichthys olivaceus*), and the channel catfish [158, 178, 241, 280]. Analysis of the pufferfish genome resulted in the discovery of TLR homologues for each of the known mammalian TLR genes with the exception of TLR4 and TLR6. Functional analysis of the different teleost TLRs has been limited to analysis of tissue expression. The analysis of the expression of the goldfish TLR showed localized expression in the spleen and kidney, and an increase in expression was observed in primary macrophage cultures 3, 6, and 24 hours after stimulation with LPS and macrophage-activation factor (MAF) [345].

Parasites possess molecular patterns capable of inducing an innate immune response through a number of different pattern recognition receptors [236]. In particular, carbohydrates on the surface of *T. cruzi* amastigotes are recognized by mannose binding lectin (MBL) that interacts with MBL-associated serine proteases (MASPs) to induce the lectin pathway of complement activation. *T. cruzi* glycophosphatidylinositol (GPI) anchors of the parasite surface molecules are PAMPs that signal through TLR-2 to induce production of IL-12, TNFα, and NO by macrophages [11]. Although the helminth *Schistosoma mansoni* has also been shown to activate TLR-2 signaling activation triggered by the parasitic worm results in the maturation of DCs that induce Th2 responses. The explanation for this dichotomy in TLR-2 induced responses to protozoan and helminth infections might be that TLR-2 is known to form heterodimers with either TLR-1 or TLR-6. Potential differences in the association of TLR-2 with other TLRs during *T. cruzi* and *S. mansoni* infection may result in the differences in T-lymphocyte responses that are observed [285].

Recently it has been reported that both TLR-2 and CD14 are important for signaling induced by *T. cruzi* GPI-anchored mucins in human monocytes. In addition the results of studies involving knock out mice for the TLR signal transduction adapter protein MyD88 imply that the parasites may stimulate macrophages through binding of yet another TLR [60]. Supporting this hypothesis is the observation that components of *T. cruzi* glycoinositolphospholipids (GIPLs) activate cells through TLR-4, and that trypanosome DNA can induce production of IL-12, TNFα, and NO. Protozoan DNA contains a significant amount of unmethylated CpG motifs and it is not unreasonable to expect that signaling may also occur through TLR-9, which is known to bind unmethylated CpG motifs of bacterial DNA [54, 155, 274]. In addition to parasite DNA, variable surface glycoproteins (VSGs), and more specifically, VSG-GPI anchors from *T. brucei*, have been shown to up-regulate gene expression and production of pro-inflammatory cytokines such as IL-1, TNFα, and IL-12. Macrophages are the source of these pro-inflammatory mediators; however, nothing is known about the receptors involved in stimulation by VSG-GPI anchors [230, 289, 356].

Similarly, for *T. borreli* infections in carp, GPI anchors and CpG motifs found in the parasite DNA are PAMPs that induce NO production. These parasite molecules are also most likely responsible for the expression of genes associated with the inflammatory response including TNFα, iNOS, and IL-1β *in vitro* [317]. In contrast, *T. danilewskyi* does not seem to activate macrophages in the same manner because NO is not produced by phagocytes in response to parasites *in vitro*, or *in vivo*. These results are interesting since the surface of the fish trypanosomes is covered in GPI-anchored mucin-like proteins that are similar to those found on the surface of *T. cruzi*, which have potent inflammatory properties [214]. One possible reason for the difference in macrophage response to the two fish hemoflagellates could be differences in GPI anchor sequences. Divergent GPI anchors from a variety of protozoans either up-regulate or down-regulate the endogenous signaling pathways of the host to the advantage of the parasite [354-5]. The PAMPs of *T. danilewskyi* may cause down-regulation of the inflammatory response by inducing the production of IL-10 by macrophages to inhibit the pro-inflammatory cytokine activity, but this hypothesis remains to be tested.

2.2.2.1.2 Effector Cells

Macrophages

Macrophages are phagocytic cells distributed throughout the vertebrate body that play a central role in the induction of both the innate and acquired immune responses to infection. In mammalian systems, the responses of these phagocytes include rapid recruitment to the site of infection, the ability to produce and respond to a variety of cytokines, the recognition of pathogens via innate immune receptors, the presentation of antigens to lymphocytes, the direct elimination of pathogens by phagocytosis, and the production of antimicrobial compounds.

Classical versus Alternative Macrophage Activation

The polarization of T-helper cells into Th1 or Th2 cytokine expressing populations is an important aspect of chronic parasite infections. The cytokine profile (Th1 or Th2) induced by a particular parasite has implications for the generation of subsets of macrophages that differ in their responses following activation. Classically activated cells that develop in a Th1 environment exhibit cytotoxic and antimicrobial functions through the production of large quantities of nitric oxide while the alternatively activated cells, which are a product of the Th2 response, produce mediators that act to dampen the inflammatory response. Th1 cells produce IFNγ that induces macrophages to metabolize the amino acid L-arginine using inducible nitric oxide synthase (iNOS), an enzyme that catalyzes the production of nitric oxide (NO). Alternatively, expression of IL-4 and TGFβ by Th2 cells inhibits the effects of IFNγ and induces macrophages to express arginase-I, an enzyme that competes with iNOS for L-arginine. Metabolism of L-arginine by arginase-I results in the production of ornithine that is then used to synthesize polyamines and proline. Thus, L-arginine metabolism and the cross-regulation of enzyme expression by Th1 and Th2 cytokines, plays a central role in determining the outcome of parasite infections [292].

In contrast to many intracellular pathogens, *T. cruzi* does not seem to induce a strict polarization towards a Th1 response throughout the course of the infection because both IFNγ and IL-10 may be induced depending on the model system [89]. In general, it seems that resistance to acute *T. cruzi* infection is associated with a Th1 response characterized by IFNγ production, and development of classically activated macrophages. Phagocytes that have been activated in this manner destroy internalized parasites by increasing the production of reactive oxygen, and more importantly, nitrogen intermediates such as NO. The ability of stimulated macrophages to produce NO, which prevents the growth of *T. cruzi*, can be inhibited by the addition of IL-10 and TGFβ. These two cytokines prevent NO production by down regulating synthesis of IFNγ by various cells of the immune system [127]. Trypanosomes that prevent production of Th1 cytokines, and induce production of Th2 cytokines, may promote their own survival by antagonizing the protective effects of IFNγ stimulation. In fact, strains of mice that are susceptible to *T. cruzi* infection produce IL-10 early in the infection whereas resistant strains do not. In addition, binding of a GPI-anchored *T. cruzi* membrane mucin, hypothesized to be involved in host cell invasion, to the surface of macrophages prevented the production of TNFα and IL-12 upon activation with LPS. A similar effect was observed for LPS-stimulated macrophages infected with *T. cruzi* indicating that alteration of cytokine production might represent a parasite evasion strategy [77]. Recently, it has been demonstrated that the *T. cruzi* specific glycoprotein, cruzipain, regulates the development of alternatively activated macrophages [131]. Concomitant with the inhibition of NO synthesis in classically activated cells, is the increased production of polyamines through the arginase pathway of L-arginine metabolism in alternatively activated macrophages. In mice, macrophages stimulated with cruzipain that are subsequently infected with *T. cruzi* develop higher numbers of intracellular parasites than unstimulated macrophages [348]. Therefore, the combination of decreased NO production, and increased production of polyamines required for trypanosome replication, indicates that molecules produced by *T. cruzi* modulate the immune response in order to facilitate parasite survival [348].

The timing of the IFNγ response to *T. cruzi* is critically restricted to the early acute phase of the infection because production of IFNγ later in the acute or chronic phases is likely not important for protection and may result in inflammation-induced pathology. In order to prevent excessive tissue damage and immunosuppression, IL-10 and TGFβ down-regulate the host response to prevent overproduction of NO, IFNγ, and IL-12 as the disease progresses [89]. Control of the NO response to the parasites is important in the chronic phase of infection, when most of the blood and tissue-dwelling parasites are removed by the acquired immune response, because NO generates impaired lymphocyte responses and immunosuppression [1].

For the extracellular African trypanosomes, resistance and control of the infection is associated with development of a Th1-type inflammatory response in the early stages of the infection, whereas the production of the Th2 cytokines IL-4 and IL-10 are important in the late acute and chronic stages [76]. Classically activated macrophages develop as a result of parasite induced IFNγ production, as well as phagocytosis of parasites, and are able to produce NO and TNFα to limit the development of the trypanosomes. Although a number of species of extracellular trypanosomes have been shown to be susceptible to NO *in vitro*, this mechanism may not be involved in limiting parasite growth *in vivo*. *In vivo*, parasites proliferate in the vicinity of activated macrophages and developed a number of ways by which they protect themselves from nitrosative and oxidative stress. These mechanisms include the production of trypanothione, the trypanosome equivalent of the antioxidant glutathione [370]. Therefore, induction of NO by *T. brucei* might represent a mechanism by which the parasites induce immunosuppression in order to establish a persistent infection. During the acute infection, there appears to be a role for Th2 cytokines, such as IL-10 produced by alternatively activated macrophages, in regulating the initial Th1-mediated inflammatory response. While resistance to African trypanosomes was dependent on IFNγ production in the mouse model [157], hosts that were unable to synthesize IL-10 died from inflammation-induced shock [256, 336]. Alternatively activated macrophages also modulate the development of a protective, specific, antibody response that is critical for elimination of parasites later in the infection by producing IL-4, down-regulating IFNγ production (by producing IL-10 and TGFβ), and preferentially attracting Th2 cells [260].

The activities of both classically and alternatively activated macrophages contribute to the immunosuppression that is associated with African trypanosome infections. The production of NO and prostaglandins by classically activated macrophages inhibit T-cell responses to mitogens and antigens that are unrelated to the parasite [90, 140]. Trypanosome-specific T-cell activity is depressed as a result of IL-10 production by alternatively activated macrophages [225, 327]. Similar to *T. cruzi*, the stimulation of alternatively activated macrophages involving the switch from iNOS mediated L-arginine metabolism to the arginase pathway favors the persistence of the African trypanosome infection. The consumption of L-arginine by arginase, and the production of polyamines contribute to parasite growth, and allow the parasites to avoid the toxic effects of nitric oxide by providing the precursors for trypanothione synthesis [260, 385].

The production of NO during *T. borreli* infections in carp *in vivo* is associated with increased susceptibility to the infection resulting in higher numbers of circulating parasites, and higher host mortality. Despite the fact that *T. borreli* induced a trypanostatic NO response *in vitro*, the results of *in vivo* experiments suggest that NO induces immunosuppression in naturally infected carp [320]. These results are similar to what has been observed for *T. brucei* infections in mice. In fact carp lymphocytes are also susceptible to NO, while the granulocytes are protected by high intracellular concentrations of the antioxidant glutathione [321]. Control of *T. borreli* in the chronic stage of the infection was associated with the production of Th2 cytokines. Failure to control the inflammatory response (uncontrolled production of NO and TNFα) resulted in increased pathology (*e.g.* anemia) that is often associated with *T. borreli* infections in fish. Based on the profile of pro-inflammatory cytokine up-regulation during *T. borreli* infection, and the observation of NO production *in vitro*, *T. borreli* may be considered to facilitate development of classically activated macrophages. Nothing is known about the nature of cytokines released in response to *T. danilewskyi* infection. However, it is known that NO is not produced *in vitro* or *in vivo* by carp macrophages [320]. Although there is no conclusive evidence to support the existence of the two states of macrophage activation in fish, it would be interesting to determine whether *T. danilewskyi* infection induces the development of alternatively activated macrophages. The ability of the parasites to proliferate in the presence of macrophages, and macrophage supernatants, might suggest that the cells are producing polyamines through the arginase pathway that contribute to parasite growth [42]. An analysis of the cytokines produced during *T. danilewskyi* infections, in addition to a comparison of arginase and iNOS expression, would be a first step in distinguishing an alternate pathway of macrophage activation during trypanosome infection in fish.

Phagocytosis

Phagocytosis by macrophages and granulocytes is an important characteristic of the innate immune response and this activity can be enhanced by the opsonization of targets with antibody or complement. The act of phagocytosis results in macrophage activation that triggers the production of reactive oxygen and nitrogen species that also have antimicrobial properties. The role of phagocytosis in the control of African trypanosomes, as well as in the control of trypanosomes infecting mice (*T. musculi*) and rats (*T. lewisi*), was established in the early 1980s. Phagocytosis by granulocytes is recognized as the major effector mechanism that culminates in the elimination of the parasites from the blood following the acute phase of the infection [332]. Greater numbers of activated macrophages appeared during the plateau phase of *T. musculi* infection in mice. Activation of macrophages was likely a direct result of phagocytosis of trypanosomes, since these phagocytic cells appear relatively late in the infection (12-14 days post infection) [381]. Phagocytosis was dependent on both the presence of specific IgG1 and IgG2a antibodies, and was enhanced by the opsonization of trypanosomes with cleavage products of complement component three (C3) [4, 226, 349, 383]. Following phagocytosis, the parasites are destroyed within the phagolysosome, probably by a cocktail of lytic enzymes and other reactive oxygen and nitrogen species [138, 384]. Paradoxically, uptake of parasites by macrophages and the subsequent macrophage antimicrobial responses, contribute to the immunosuppression that is a characteristic of trypanosome infections [140].

While phagocytosis of trypanosomes by macrophages or granulocytes has not been observed for *T. danilewskyi* infections, ingestion of foreign material remains an important innate effector mechanism in fish [259]. In fact peritoneal and kidney macrophages from fish that have recovered from a natural infection, or that have been vaccinated, engulf another related fish hemoflagellate, *C. salmositica* [211, 402].

NK cells

Since their initial identification and characterization in the early 1970s, natural killer (NK) cells have attracted interest as key effector cells of the immune system. NK cells have a wide variety of targets including tumor, and virally infected cells. Unlike cytotoxic T-cells, targeting of NK cells to pathogens is not antigen specific. NK cells are large, non-phagocytic, granular lymphocytes characterized by the expression of FcγRIII (CD16) on almost all cells of this type. Targeting of NK cells is regulated by competing inhibitory and activating signals that arise through direct interaction with target cells. The interaction of NK cells with their targets is classically defined by a mechanism known as "missing self" recognition. In this model, signals provided through the interaction of activating receptors are inhibited due to the concurrent binding of inhibitory receptors to the MHC class I molecule normally expressed on the surface of host cells. In the absence of the inhibitory MHC class I signal, activation signals propagate throughout the cell initiating killing activity [162]. The interaction between MHC class I inhibition, and the activating signals provided by the target cells do not always favor inhibition because engagement of certain activating receptors can override MHC class I inhibition [154].

Studies of the role of NK cells in the control of kinetoplastid parasites have been directed toward species with an intracellular stage (*e.g. Leishmania, T. cruzi*) because NK cells are known to participate directly in the initiation of the Th1-type immune responses by producing IFNγ. In many cases IFNγ is required for protection against intracellular pathogens. However, since NK cells are also directly cytotoxic to the free stages of these parasites, it seems likely that they may play unidentified roles in the innate immune response to a number of other extracellular parasites as well [213].

In fish, two populations of cells that resemble NK cells of higher vertebrates in terms of form and function have been identified, the non-specific cytotoxic cells (NCCs) and the cytotoxic NK-like cells.

Non-specific cytotoxic cells

The presence and characterization of a novel cytotoxic cell population in the channel catfish was described in the mid-1980s. These cells, called non-specific cytotoxic cells (NCCs), are unique in their ability to lyse various transformed human and mouse cell lines. The lysis of target cells requires direct cell-cell contact through a mechanism thought to be similar to that of mammalian NK cells. Being small and agranular, these cells are morphologically similar to monocytes. NCCs are found in the fish anterior kidney rather than the blood and functional similarities to mammalian NK cells have led researchers to postulate that NCCs might represent the evolutionary precursor of NK cells [101-3]. NCCs have been identified in numerous teleost species including rainbow trout, carp, damselfish (*Dascyllus albisella*), and tilapia (*Oreochromis* spp.) [106, 139, 238]. The identification of the cells in different species of fish was assisted by the development of a monoclonal antibody that could specifically target NCC populations. The antibody, mAb 5C6, binds to a membrane protein on the surface of catfish NCC. Incubation of catfish NCC with mAb 5C6 prevented the cytotoxic activity of these cells towards a variety of target cell lines including NC-37, MOLT-4, YAC-1 and HL-60. The NCC-specific antibody appears to inhibit lysis by interfering with conjugate formation between the effector cell and the target cell [104]. Interestingly, it was also discovered that mAb 5C6 binds a large proportion of human NK cells, and that the binding of the antibody inhibited their ability to kill a number of transformed cell lines by preventing conjugate formation [104].

In addition to the ability to lyse transformed cell lines, NCCs appear to participate in the immune response against protozoan parasites. Graves and colleagues [137] demonstrated *in vitro* killing of *Tetrahymena pyriformis*, an opportunistic ciliated protozoan parasite, by catfish NCCs. Specific *T. pyriformis* antibodies enhanced NCC killing of the parasites, probably by immobilizing the cilia and not through antibody-dependent cell cytotoxicity. One mechanism of NCC-mediated parasite killing may involve the induction of apoptosis in the parasites through a Fas ligand-Fas receptor interaction [175]. Through binding deletion experiments, it was found that another protozoan parasite, *Ichthyophthirius multifiliis,* had a similar target antigen to *T. pyriformis*. In addition, infection with *I. multifiliis* caused a shift toward increased percentages of NCCs in the peripheral blood [137]. A monoclonal antibody was used to identify a 40-50kD molecule from the lysates of the protozoan *T. pyriformis* that was recognized by catfish NCCs. The amino acid sequence of the protein, named NKTag, has no significant homology with any known sequence. However, soluble or purified NKTag inhibited NCC-mediated lysis of target cells. Immunofluorescence studies have revealed that a related protein was expressed on several transformed mammalian cell lines [177, 207]. These results highlight the importance of the NKTag epitope to the cytotoxic response of NCCs and possibly other NK cells. Immunoprecipitation experiments using mAb5C6 (anti-NCC antibody) resulted in the identification of a protein of 34 kDa named NCC receptor protein 1 (NCCRP-1) [104, 174, 176]. The engagement of NCCRP-1 by NKTag was responsible for the recognition and killing of target cells by NCCs [105, 174, 177, 207].

Cytotoxic NK-like cells

In addition to the NCCs, which may represent an evolutionary precursor of mammalian NK cells, catfish also possess another spontaneous cytotoxic cell type that has been labeled NK-like cells. Recently, new clonal NK-cell lines have been identified from alloantigen-stimulated catfish lymphocytes that share functional and morphological similarities to mammalian NK cells, but that are distinct from NCCs [333]. The catfish NK-like cells are large and granular, which is similar to mammalian NK cells, but unlike NCCs. These NK-like cells lysed allogenic target cells but failed to express immunoglobulin or T-cell receptors, excluding the possibility that they are B or T-lymphocytes. In addition, these cells do not stain with Sudan Black B and are non-specific esterase-negative indicating that they are not neutrophils or macrophages. Interestingly, some NK-like clones express the FcεRγ chain and bind IgM through a putative FcμR [333-4]. Perhaps the most significant finding was that mAb 5C6 (anti-NCC antibody), failed to recognize these cells, suggesting that they are not NCCs. Although all of the NK-like cell lines investigated exhibited cytotoxicity toward allogenic targets, the specificity varied between clones. Some clones killed several different allogenic cell types equally, while others demonstrated a marked specificity for a particular cell type. Killing of target cells by catfish NK-like cells appears to be mediated through the induction of apoptosis [333]. The precise mechanism of the induction of apoptosis is not known; however, the ability to inhibit NK-like cytotoxicity by chelating extracellular calcium ions indicates that secretory pathways featuring perforin and granzyme molecules are involved [333]. The ability of the catfish NK-like cells to cause spontaneous or antibody dependent lysis of any fish pathogens, or pathogen infected cells, has not yet been determined.

NK cells are a major source of IFNγ in the early stages of *T. cruzi* infection in mice, which drives the cytokine profile towards a protective Th1 response involving production of additional IFNγ as well as IL-2. Only live *T. cruzi* stimulate IFNγ production, suggesting that the induction of cytokine production is caused by penetration of parasites into host cells, or by parasite excretory-secretory products. It is believed that the parasites initially stimulate the production of IL-12, which in turn activates NK cells to produce IFNγ that mobilizes the anti-microbial responses of macrophages [14]. The production of IL-12 was critical for the establishment of a Th1-response, since IL-12 knockout mice are extremely susceptible to infection [374]. Removal of IFNγ, or neutralization of NK cells, resulted in increased susceptibility to infection and an increase in IL-10 production that inhibited trypanocidal effects. IFNγ has been implicated in the control of *T. cruzi* infections in the acute stage by inducing macrophages to produce TNFα, and activating iNOS.

There seems to be little role for IFNγ in the control of chronic *T. cruzi* infection. In fact sustained concentrations of IFNγ and IL-12 in the serum may be responsible for the tissue inflammatory reactions that are associated with the pathology induced by chronic *T. cruzi* infections [14]. Although the production of IFNγ seems to be important for control of *T. cruzi* infections, the levels of this cytokine tend to be the same in susceptible and resistant mice. On the other hand, the concentrations of IL-10 were lower in resistant mice. In conclusion, although IFNγ production may be important, the differential production of IL-10 determines the patterns of susceptibility and resistance to *T. cruzi* [62].

In addition to having direct cytotoxic effects, NK cells also have a role in regulating B-cell activation. *In vitro*, T-independent antigen-induced B-cell responses require the presence of NK cells, which play an accessory role by producing cytokines and interacting directly with B-cells. Glycoinositolphospholipids (GIPLs) are major glycolipids on the surface of *T. cruzi* that induce B-cells to produce immunoglobulins in the absence of T-cells and NK cells. In addition, GIPLs also have a direct stimulatory effect on NK cells, and therefore these parasite molecules may be responsible for the activation of NK cells during *T. cruzi* infections. Addition of NK cells to B-cells stimulated by parasite GIPLs leads to an increase in immunoglobulin production that can be further enhanced by the addition of exogenous IL-2 [75]. Additional studies have confirmed the ability of NK cells to stimulate immunoglobulin production by B-cells isolated from trypanosome infected mice through a mechanism involving direct cell contact. Thus, NK cells seem to be involved in the generation of the polyclonal antibody response associated with *T. cruzi* infections [74].

Removal of NK cells increases susceptibility to infection and the administration of cytokines that stimulate NK cell activity increased resistance to *T. cruzi*. It is unclear whether NK cells kill infected cells and free parasites directly, or whether they produce mediators that induce other cell types to destroy the pathogens. Cytotoxic cells such as NK cells and cytotoxic T lymphocytes utilize effector molecules including perforins, granzymes, and the Fas-ligand to kill targets. Perforin is a Ca^{2+} dependent, pore-forming protein that inserts into the target cell membrane, disrupts permeability, and eventually leads to cell lysis. All of the life cycle stages of *T. cruzi* are resistant to the pore-forming protein perforin produced by cytotoxic lymphocytes. Resistance of the parasites was proposed to be due to their ability to prevent transmembrane pore formation. Since the parasites possess their own pore-forming complement component-like protein, resistance to perforin may be associated with the resistance of the parasites to their own pore-forming molecule. Since NK cells induce killing of *T. cruzi*, and since perforin knockout mice were susceptible to *T. cruzi* infections, it is clear that there must be other mechanisms aside from perforin involved in target cell lysis. A recent study has shown that NK cells activated *in vivo* during *T. cruzi* infection, perform contact-dependent destruction of parasites that was probably mediated by exocytosis of lytic granules and not dependent on perforin. When free parasites were incubated with NK cells, contact formation was followed by a loss of trypanosome mobility and membrane integrity. The same series of events did not occur when the parasites were co-incubated with T- cells. The trypanocidal effect of the NK cells was dependent on the production of IL-12 early in the infection [213]. Some researchers have argued that NK cell cytotoxicity is not important for control of *T. cruzi* infections because mice that are deficient in α and β IFN (responsible for the early control of NK cytotoxicity) are no more susceptible to infection than normal mice. In addition, others have shown that cells expressing NK cell markers (NK1.1) were protective only in the presence of a functional thymus [374].

Early studies of the effects of NK cell activity on the course of extracellular trypanosome infections involved the rodent parasite *T. musculi*. NK cell activity in mice infected with *T. musculi* does not correlate with the elimination phase of the infection [6]. Although NK activity is high early in the infection (3-4 days), there was a rapid decline in activity to sub-normal levels that persisted throughout the parasite elimination phase [6]. The early activity and accompanying proliferation of NK cells was believed to mediate control of the infection through the activation of peritoneal macrophages because NK cells were unable to directly participate in the killing of *T. musculi* [7]. It is not known whether the decrease in activity that was observed in both the spleen and peritoneal exudate cells occurred as a result of suppressor cells, or suppressor compounds, of host or parasite origin [6]. Because the rodent trypanosomes are host-specific, it is possible that *T. musculi* has evolved mechanisms to prevent lysis by NK cells in mice. Although murine NK cells displaying heightened cytotoxic activity were not able to inhibit the normal course of *T. musculi* infection in mice they were capable of spontaneously killing *T. lewisi* and *T. cruzi* [5]. These results suggest that *T. musculi* may produce inhibitory substances that render them resistant to the cytotoxic cells. In fact it was also shown that the addition of live parasites, or *T. musculi* extracts, prevented the normal cytotoxic response of murine NK cells to target cells while extracts of *T. lewisi* did not. The results of these studies suggest that host specificity may play a role in determining whether the parasites are susceptible to NK cell activity. *T. musculi* and *T. lewisi* are particularly host specific and are well adapted to their respective hosts (mice and rats) while *T. cruzi* has a much wider host range [5].

2.2.2.2 Acquired Immune Response

Both B and T lymphocytes are required for control of many types of infections in fish and mammals including viruses, bacteria, protozoans, and multicellular pathogens. Both cell types are dependent on the recognition of microbial molecules by antigen presenting cells (APCs) of the innate immune system. APCs present foreign peptides on their surface, in the context of MHC class II, to T-cells and B-cells bearing receptors specific for a particular combination of peptide and MHC. This interaction induces clonal proliferation of the activated lymphocytes, the subsequent production of immunomodulatory cytokines, and activation of immune effector mechanisms. Stimulated B-cells produce antibodies that are involved in neutralization of viruses and toxins, activation of the classical complement pathway, and antibody-dependent phagocytosis. T-helper cells produce cytokines that regulate the immune response and aid in the generation of reactive B- and T-cell populations while cytotoxic T-cells play a role in lysis of *T. cruzi* infected cells, and in the production of immunomodulatory cytokines.

2.2.2.2.1 T-cell responses

T-cell populations in mammals are heterogeneous and consist of a number of different subsets of cells characterized by their function, and specific sets of receptors and ligands present on the cell surface. Most T-cells express a T-cell receptor (TCR) and either CD4 or CD8 on their surface. CD4 is a marker for T-helper cells, which are involved in the production of cytokines such as IL-2, that stimulate additional development of various T-cell subsets. T-helper cells are important in controlling the type of immunity generated to an infection, since they can become polarized in terms of their cytokine expression profile to induce either a Th1-type (involving IFNγ) or a Th2-type (involving IL-4, IL-5, IL-10) acquired immune response [200]. As discussed previously, the Th1/Th2 cytokine balance is crucial in determining resistance or susceptibility to infection. T-helper cells are activated by APCs (macrophages, dendritic cells, and B-cells) expressing peptide-bound major histocompatibility complex (MHC) class II molecules. The MHC class II-peptide complex interacts with a compatible TCR and its CD4 co-receptor on the T-helper cell leading to MHC class II-restricted antigen presentation, clonal expansion of select T-cells, and cytokine production [200]. $CD8^+$ T-cells are cytotoxic cells that have the ability to directly kill altered-self and foreign cells that they recognize by interacting with specific molecules expressed on the surface of leukocytes and target cells. These molecules include the TCR and the TCR co-receptor CD8 on the cytotoxic T-lymphocytes, as well as the major histocompatibility complex (MHC) class I molecules expressed on the cells with which T-cells interact. To lyse a target cell the TCR on the surface of the lymphocyte must recognize a compatible peptide bound to MHC class I. Cytotoxic T-cells cause cell lysis by inducing apoptosis through the activity of the contents of cytolytic granules, or through binding of Fas by Fas ligand. Another unique characteristic of T-cells is their ability to respond to a secondary infection without stimulation from APCs, such as macrophages, as part of the immunological memory associated with the adaptive immune response [200].

Both specific and non-specific cytotoxic cells have been observed in fish [119]. The non-specific activity has been attributed to NK-like cells and NCCs while specific lysis of targets occurs as a result of a population of IgM^-, $CD8^+$, TCR^+, MHC class I-bearing cells that probably represent cytotoxic T-cells. Positive identification of these cells in fish has been hindered by the lack of specific markers and monoclonal antibodies that are available for the study of mammalian cell-mediated immunity. For example, a population of CD4$^+$ T-helper-like cells has yet to be defined in fish although identification of a few of the critical markers for the cytotoxic T-cell population (TCR, CD8$^+$, MHC class I), in conjunction with some *in vivo* and *in vitro* functional studies, strongly suggest their existence as a distinct cell population. A number of genes involved in T-cell biology have been identified from fish genome projects and expression libraries. However, the identification and function of the molecules at the protein level is still in the early stages [119]. Fish cytotoxic cells may be induced by many of the same cytokines involved in the generation of mammalian cytotoxic lymphocytes (IL-2, IL-6, IL-12) since the gene sequences for these molecules have recently been identified. Functionally, they respond to T-cell mitogens including concanavalin A, and are able to lyse a variety of target cells from different heterologous and autologous sources *in vitro*. Interestingly, the response to T-cell mitogens is inhibited at low temperature, providing a partial explanation for the suppression of the acquired immune response of fish at cold temperatures [67]. Killing of target cells by cytotoxic lymphocytes has also been shown to occur by induction of apoptosis. *In vivo* experiments have shown that fish have the ability to reject allografts, mount delayed-type hypersensitivity responses, and are susceptible to graft-versus-host disease, all of which are common models for studying specific cell mediated responses in mammals [119].

The acquired immune responses of the higher vertebrates are important in the control of trypanosome infections, particularly as the disease enters the chronic phase, because the parasites have been shown to induce immunosuppression of T-and B-lymphocyte activity in the early acute response. Experimental trypanosome infections in mice lead to polyclonal proliferation of B- and T-cells in the hematopoietic organs that gives way to a phase of unresponsiveness and generalized immunosuppression [140, 247]. The causes of the immunosuppression have been shown to be both parasite and host induced. More specifically, a number of parasite products alter cytokine profiles and induce the production of nitric oxide (NO) and prostaglandins by macrophages. Both NO and prostaglandins are associated with the inhibition of lymphocyte proliferation. Parasite molecules that are involved in direct and indirect immunosuppression include, but are not limited to, *T. cruzi trans*-sialidase, sialylated mucins, and GIPLs. For *T. brucei* infections, GPI anchors and the trypanosome lymphocyte triggering factor (TLTF) are important stimulators of NO production [90, 140]. Apoptosis of T-cells may also play a role in immunosuppression and exacerbation of the *T. cruzi* infection. Uptake of apoptotic cells induces anti-inflammatory responses including production of prostaglandin and TGFβ by macrophages [90]. In fact phagocytosis of apoptotic cells is thought to drive alternative activation of macrophages resulting in down- regulation of IFNγ production and prolonged parasite persistence. The parasite cysteine protease, cruzipain, mimics the effects of phagocytosis of apoptotic lymphocytes since it also leads to alternative activation of macrophages. Treatments that prevent apoptosis of $CD8^+$ T-cells may be therapeutic because blocking apoptosis leads to an accumulation of memory $CD8^+$ T-cells [275].

In spite of the immunosuppression caused by the parasites during the acute phase of the infection, both $CD4^+$ and $CD8^+$ effector T-cells are required for control of *T. cruzi* infection during the acute and chronic phases of infection, and may also be important for vaccine-induced immunity [89, 360]. A slightly biased Th1-type response is critical in the production of antibodies, activation of phagocytes, and induction of T-cell help for other T-cells [16]. Infection of mice lacking $CD8^+$ lymphocytes, or of mice that are deficient in T-cell components, resulted in exacerbation of the disease including an increase in parasitemia and mortality [362]. In addition, transfer of parasite specific $CD8^+$ cells can provide significant protection from infection [400]. A role for $CD8^+$ T lymphocytes in the control of *T. cruzi* is not surprising since the intracellular location of the replicating forms of the parasite would result in the presentation of parasite peptides in association with MHC class I. Activation of T-cells is required for the production of IFNγ, mainly by $CD4^+$ cells, which has been associated with resistance to both primary and secondary parasite infections [161]. IFNγ stimulation of macrophages leads to production of TNFα and

NO that aid in the destruction of intracellular parasites [127]. Parasite specific CD8-bearing T-cells are also able to directly target and lyse infected cells [233]. In some environments such as the host muscle and heart tissue, $CD8^+$ effector mechanisms are impaired, resulting in increased pathology due to parasite persistence [208]. Parasite T-cell targets include various members of the *trans*-sialidase family, as well as the unexpected finding of the response of $CD8^+$ cells to trypanosome intracellular proteins, including heat shock proteins and paraflagellar rod proteins [295, 407]. The identification of target T-cell epitopes is important because they make excellent vaccine candidates [233, 293]. The $CD8^+$ T-cell response to a multitude of parasite antigens has been implicated in the slow and sub-optimal development of the acquired immune response associated with the persistence of *T. cruzi* infections in mice. The presence of large multi-gene families, such as the *trans*-sialidase family in *T. cruzi* (>800 genes) was also associated with the evasion mechanisms of other parasites including the well-studied variable surface glycoprotein genes of *T. brucei* [2, 290]. Although any one of the trypanosome *trans*-sialidase molecules may be recognized as a T-cell epitope, the presence of an enormous number of similar variants could compromise the quality and magnitude of the response [233].

T-lymphocyte activation is an important component of the immune response to African trypanosomes and is manifested by a delayed-type hypersensitivity response suggesting the enhancement of the activity of T-helper cells [118]. T-helper lymphocytes were also implicated in the B-cell responses involved in eliminating *T. musculi* from the mouse host. T-helper cells provide assistance with the generation of anti-parasite antibodies; however, there is little apparent role for cytotoxic T-cells in generating immunity [164]. In fact for *T. brucei* infections in rats it was shown that depletion of $CD8^+$ T-cells resulted in suppression of parasite growth and increased host survival [23]. These results are in contrast to the importance of both T- cell subpopulations in the control of *T. cruzi* infections. The relative importance of $CD8^+$ and $CD4^+$ T-cells for controlling *T. brucei* infection was determined using mice deficient for each cell population. Infected mice lacking $CD8^+$ T-cells exhibited enhanced resistance to the parasite and enhanced IL-4 production following mitogen stimulation. This observation is likely the result of a decrease in IFNγ production that: (1) acts as a growth factor for *T. brucei*; and (2) contributes to the production of pro-inflammatory cytokines that inhibit the production of other cytokines involved in generating a protective antibody response [314]. Resistance to infection was also slightly enhanced in CD4$^-$ mice but not to the extent of the CD8$^-$ mice. T-cell-dependent, parasite-specific IgG responses were produced in CD8$^-$mice but not in CD4$^-$ mice [314]. Since *T. brucei* is an extracellular parasite it was expected that a protective antibody response would be associated with resistance to infection. The slightly enhanced resistance to *T. brucei* in mice lacking CD4$^+$cells suggests that production of T-cell-dependent antibodies was not required for control of extracellular trypanosomes in mice [314]. Mice generate antibodies to variable surface glycoproteins in the absence of T-helper cells; however, the optimal response is to T-cell dependent antigens [308].

While the identification of T-cell subsets in fish that are similar to those found in mammals is still in its infancy, the identification of genes specific to T-cells is increasing as a result of genome mining. Soon it will be possible to identify proteins and study the function of these cells in their resting state, and in response to infection. To date, a few experiments have suggested the presence of a T-helper-like lineage involved in the B-cell responses to T-dependent antigens [376]. Also, putative cytotoxic cells cause lysis of virally infected cells and may be responsible for the immunity generated following vaccination with viral extracts [87].

NKT cells

A special subset of T-cells are the NKT cells. These cells are different from conventional T-cells and NK cells because they possess receptors of both cell types (T-cell receptor (TCR), NK1.1 receptor). They differ from the majority of T-cells in that they are limited in the diversity of the TCR and respond to glycolipid antigens that they present using CD1d rather than MHC molecules [132]. They secrete large amounts of either IFNγ or IL-4 and their function may be to provide immediate help for Th1 (IFNγ) or Th2 (IL-4) responses. In doing so, these cells represent a cellular link between the innate and acquired immune defenses, and they are able to respond rapidly to regulate anti-inflammatory and anti-pathogen responses [132]. NKT cells have direct cytolytic activity, can stimulate NK cell responses through the production of IFNγ, and have also been associated with the prevention of self-damaging responses [132].

A growing body of evidence suggests that NKT cells play an important role in the regulation of immunity to a variety of disease-causing organisms [153]. During *T. cruzi* infections, parasite expressed glycophospatidylinositol (GPI) stimulates IL-12 production that in turn stimulates NK and NKT cells. The role of NKT cells in limiting *T. cruzi* infections in mice is suggested by the fact that NKT deficient mice have increased parasitemia, and poor antibody responses to GPI modified surface proteins. Once they have become activated, NKT cells undergo activation-induced cell death. As a result, their ability to inhibit the self-damaging effects associated with chronic trypanosome infection through the production of inhibitory cytokines such as IL-4 is impaired [15, 248]. NKT cells act to decrease parasitemia during the acute phase of the infection and can be stimulated by IL-12 and/or by binding to the TCR or NK1.1 receptors on the cell surface. The parasites stimulate IL-12 production that will activate NKT cells to produce IFNγ, and NKT cells may also directly lyse trypanosomes [97]. Recently it has been determined that although the activity of NK cells could be regulated by NKT cells, the activation and protection provided by NK cells in the acute *T. cruzi* infection was not dependent on NKT cells. Interestingly, regulation of the size of the liver NK cell population was NKT cell-dependent, but the mechanism by which the NKT cells perform this function is unknown [96]. NKT cells act very early in the infection (within a day) since they either disappear, or become less responsive, following the initial days of infection. A recent study [15] has shown that in the later stages of the acute infection the NKT population increased in resistant strains of mice. During this stage of infection, NKT cells may play a part in down-regulating the production of INFγ by secreting inhibitory cytokines. Thus, NKT cells seem to play a dual role during *T. cruzi* infections since they can stimulate a protective Th1 response early in the infection, and also have the ability to prevent the inflammatory response from causing excessive tissue damage in the late acute and chronic stages of infection [15]. The results of recent studies have led to the identification of a mechanism by which the same cell type augments and limits certain aspects of the anti-parasite immune response. Two subsets of NKT cells have been identified, one set expresses an invariant T-cell receptor (iNKT) and the other set utilizes a variable repertoire of $\alpha\beta$ and $\gamma\delta$ TCR genes (vNKT). Mice deficient in iNKT cells suffer from an inability to control the inflammatory response to *T. cruzi* and display greater morbidity and mortality than wild type mice or mice deficient in both subsets of NKT cells. Therefore, for the first time it has been demonstrated that two subsets of cells have separate functions in the regulation of the immune response to the same pathogen. The vNKT cells seem to promote the inflammatory response to the parasites while the iNKT cells seem to be necessary to down-regulate the inflammatory response in the acute phase [95]. The precise role of NKT cells in the chronic phase of *T. cruzi* infection is not clear. The results of initial studies suggested that the activity of NKT cells in the chronic phase of the *T. cruzi* infection in mice augments the antibody response. It seems that the properties of an individual NKT cell response will affect the parasitemia, the intensity of the chronic inflammatory response, and the potential outcome of the disease [97].

Other authors argue that there is no role for NKT cells in the generation of protective immunity during *T. cruzi* infections. They suggest that NKT cell activity could result in the impaired induction of CD8$^+$ T-cell responses that provide vaccine-induced protective immunity. These findings are in direct contrast to the results of investigations of other pathogen infections where NKT cells play a role in generation of protective immune responses. The differences in the results regarding *T. cruzi* infection may have to do with the strains of parasites and hosts used for the experiments, the inoculation routes used for infection, and administration of NKT cell-activating substances. Alternatively, it is possible that NKT cells have different roles during different immunological and disease scenarios by virtue of their ability to produce a number of different cytokines that have contradictory roles [248]. To date, NKT cell populations have not been identified in fish.

2.2.2.2.2 B-cell responses

B-cells, in addition to T-cells, are the cell types that characterize the classical definition of acquired immunity. In mammals, these lymphocytes are generated in the bone marrow and although their main roles are antigen presentation and the production and secretion of an extensive variety of antibody molecules, they are also involved in interactions with macrophages, dendritic cells, and T-cells. The ability of B-cells to respond and produce antibodies to a wide array of specific antigens is due to the mechanism of B-cell receptor (BCR) V-D-J gene segment rearrangement, as well as the processes of class switching and somatic hypermutation [200]. The latter two mechanisms are important in improving the affinity of a given antibody for its antigen, and in tailoring the acquired immune response to a particular type of infection. Affinity maturation through mutation and class switching is also thought to be critical for the generation of memory B-cells [184].

In the lymphoid tissues, antigen-presenting cells interact with B-cells through the BCR and peptide-bound MHC class II on the APC. Following antigen presentation, B-cells can become activated on their own, or with the assistance of T-helper cells. B-cell antigens can be divided into two groups, thymus-dependent and thymus-independent. Thymus-dependent antigens tend to be soluble molecules that do not bind the BCR strongly, and require 'help' from $CD4^{+}$T- lymphocytes. Thymus-independent antigens cause extensive cross-linking of the BCR and therefore stimulate B-cells without T-cell help. Although B-cell responses to T-independent antigens are generally non-specific for certain antigens, such as bacterial LPS, administration of low concentrations will result in specific antibody production. T-independent antigens that are present on the surface of a pathogen tend to include repetitive sequences that can bind multiple receptors [387]. In general, the response to T-independent antigens is weaker than the response to T-dependent antigens since the processes of affinity maturation, isotype switching, and generation of memory cells are associated with the response to T-dependent antigens. Once a particular B-cell is activated through the recognition of an antigen specific to its BCR in the context of co-stimulatory molecules it will proliferate and the members of the clonal population will differentiate into antibody-secreting plasma cells, and memory cells [200]. Somatic mutations occur during B-cell differentiation in which the affinity of the BCR for the antigen increases (affinity maturation). In addition, class switching, involving a change in the heavy chain constant region of the immunoglobulin while maintaining antigen specificity, alters the way in which antibody-dependent cellular processes occur during the immune response. There are two B-cell populations in mammals, B1 cells found in the lymphoid tissues express IgM and co-receptors for antigen presentation (CD40), and respond to T-cell dependent antigens. The B2 cell populations expressing IgM, IgD, and CD5, are located in the peritoneal cavity, and are involved in the early immune response to T-independent antigens [276].

In addition to their role in antibody secretion and the generation of memory, B-cells can also act as APCs by processing antigen bound to the BCR and expressing it in the context of MHC class II for presentation to T-helper cells [200]. It is becoming more apparent that the activity of B-cells is intimately connected with the innate immune system. For example, it has been determined that a second signal required for antibody secretion by certain populations of B-cells could be provided by toll-like receptors from cells of the innate immune system [387]. The complement system has also been linked to the B-cell response and receptors for complement components have been identified on the surface of B-cells. Cleaved fragments of C3 bound to antigen on target cells induce and enhance antibody responses by binding to complement receptor 2 (CR2) on the surface of B-cells while simultaneously binding the B-cell receptor. The threshold for B-cell activation is decreased significantly in the presence of antigen and for this reason it is believed that C3d acts as a natural adjuvant *in vivo* [82].

Acquired immunity, as it is defined in the mammalian paradigm, originated in the cartilaginous fish with the appearance of the recombination activating genes (RAG 1 and RAG 2), which are responsible for generating the recombinatorial B- and T- cell receptors. Therefore, it is in this group that antibodies and the antibody dependent mechanisms of immunity first appeared. Although fish were known to possess cells demonstrating lymphocyte-like morphology, it was not until antibodies were generated toward serum immunoglobulins that a specific IgM^+ population could be distinguished from IgM^- cells. Like their mammalian counterparts, fish B-cells express immunoglobulin on their surface and secrete antibodies in response to antigenic challenge [183]. Subsequently, heterogeneous populations of B-cells and immunoglobulins were identified in carp (also described in other fish including salmon and catfish) using panels of monoclonal antibodies to serum immunoglobulins and flow cytometry. The results of these studies suggest the existence of different soluble and membrane bound isoforms of carp IgM. Functional differences for these isoforms have not been determined; however, it has been suggested that they may play a role in B-cell differentiation or in the mucosal immune response [198]. Responses of fish B-cells to antigens occurs via mechanisms similar to those described in mammals in which MHC class II-restricted antigen presentation and interaction with Ig^- cells (probably T-lymphocytes) are required for activation by T-dependent antigens. Fish B-cells can also respond to T-independent antigens on their own without the requirement of antigen presentation by macrophages [68, 376]. The temperature sensitivity of the specific immune responses of fish can be explained in part by the observation that the lymphocyte response to T-dependent antigens is inhibited at low temperature. As mentioned above, the effects of low temperature are hypothesized to occur at the level of the T-cell rather than the B-cell [67, 245]. These observations illustrate the importance of T-helper lymphocytes, and probably specific cytotoxic T-cells, in the generation of specific antibody and cellular responses to pathogenic organisms.

As described previously, fish antibodies are of lower affinity and diversity than those of the higher vertebrates and fish also seem unable to mount a typical anamnestic response (as defined in the mammalian system) to secondary antigen exposure [93]. In mammals, early studies of the requirements for generation of memory B-cells included identification of T-cell participation, and the involvement of T-dependent antigens. Later, other processes involved in memory cell development were identified such as isotype switching, and somatic mutation. As indicated earlier these latter mechanisms do not occur in fish and the lack thereof may have a significant effect on the generation of memory antibody responses in fish. It has been suggested that the B-cell proliferation, and increased antibody production, that is observed following secondary antigen exposure in some fish studies may be the result of an increase in the number of B-cells that are sensitive to antigen stimulation. This is in contrast to the change in the physiological state of the existing lymphocyte pool as observed in mammals [183]. This hypothesis has been supported by the results of immunization studies involving rainbow trout injected with an attenuated strain of *Aeromonas salmonicida*. B-cells proliferated in response to the bacteria, although not to the extent of T-cells, and the increase in cell number was determined to be due to an increase in the precursor B-cell pool rather than an increased capacity for proliferation [232]. Despite these apparent deficiencies in the B-cell responses of fish, immunization against a number of different pathogens can be achieved and results in the production of measurable quantities of antibody specific for the immunizing antigen [142, 212, 377].

While antibody responses to hemoflagellate infections in fish are important for control of the infection and prevention of disease, direct demonstration of the proliferation of B-cells in response to the whole organism or parasite products has not been demonstrated. This is likely due to the lack of immune cell markers for fish and the difficulty in obtaining pure populations of B-lymphocytes. However, specific proliferation of mixed populations of lymphocytes has been shown in response to immunization of Atlantic salmon with an attenuated strain of *Cryptobia*, and polyclonal lymphocyte activation has been demonstrated for *T. borreli* infections in carp [18, 317].

2.3 Conclusion

The immune response of higher vertebrates to kinetoplastids, and to trypanosomes in particular, has been studied in great detail. The focus of this review was the members of the genus *Trypanosoma*. One difficulty that is encountered in the interpretation of the results of the studies in this field of research is the fact that mice are not natural hosts for the two groups of trypanosomes that have been studied most intensively, *T. cruzi* and *T. brucei*. Infections in the natural hosts of these parasites are characterized by relatively low parasitemia during the acute phase followed by the development of chronic infections in which few parasites are detected in the blood. Death of the natural hosts is not a desirable outcome for parasite transmission and when death does occur it is usually as a result of secondary infection, or the inability of the host to regulate inflammation. In mice, infections with *T. brucei* or *T. cruzi* are normally characterized by high parasitemia in the acute phase that often leads to death of the host. In addition, the immune response observed to trypanosome infections in mice often depends on both the strain of parasite, and strain of host, used in the study. Despite the limitations of these animal models, it seems clear that although the hosts mobilize a variety of innate and acquired immune responses to the parasites, in many cases the trypanosomes are still able to establish long-lasting infections. Whether this is a result of immunosuppression of the host, evasion strategies of the parasite, or both is difficult to determine. The goldfish-*T. danilewskyi* model system is representative of a natural host-pathogen interaction and therefore represents a unique opportunity to study both the immune responses of the lower vertebrate host to hemoflagellate infection in more detail, and to examine the strategies used by the parasite to establish chronic infections.

CHAPTER 3

MATERIALS AND METHODS

3.1 General Procedures

3.1.1 Fish

Goldfish, *Carassius auratus* (L.), were purchased from either Ozark Fisheries Inc. (Southland, MO, USA) or, Grassy Forks Fisheries (Martinsville, IN, USA). The fish were fed *ad libitum* and housed in tanks with a continuous-flow water system at 20°C in the aquatic facility of the Biological Sciences Centre, University of Alberta under a 14 hour light:10 hour dark photoperiod. Prior to manipulation (bleeding, injection, clipping) fish were anaesthetized by immersion in a solution of tricaine methane sulfonate. When necessary, fish were marked by fin clipping.

3.1.2 Bleeding of fish and preparation of fish serum/plasma

Fish serum was obtained by bleeding from the caudal vein using a syringe fitted with a 23-guage or 25-gauge needle (for fish <14 cm in total standard length). After allowing the whole blood to clot for at least 4 hours at 4°C, it was centrifuged (1560 x *g*, 30 minutes) and the supernatant was collected. Serum used for the maintenance of cell cultures was heat-inactivated (56°C, 30 minutes), filter sterilized (Millipore 0.22 μm), and frozen at -20°C until needed.

For immunoassays, small quantities of plasma were obtained from immunized fish. After determining the red cell volume, the glass capillary tube was etched and broken above the buffy coat. Plasma was then extracted from the tube using a syringe fitted with an adaptor for a capillary tube (BioRad). Samples were stored at -80°C until needed.

3.1.3 Parasites

Trypanosoma danilewskyi (strain TrCa) was isolated from a crucian carp, (*Carassius carassius*) by Dr. J. Lom in 1977. The parasites were obtained from Dr. P.T.K. Woo, University of Guelph (ON, CAN). Trypanosomes used for all assays were obtained from stock cultures that were 7 days old. Stock cultures were maintained at 20°C in trypanosome culture medium (Table 3-1) containing 10% goldfish serum. The cultures were passaged every 6-7 days by subcultivation in fresh medium at a ratio of 1:10 (v/v).

3.1.4 Purification of parasites from the blood

Trypanosomes were purified from the blood using a modified protocol developed for *Trypanosoma brucei* [63]. Briefly, tri-sodium citrate was added to the blood samples to prevent clotting, the sample was centrifuged for 10 minutes at 750 x *g*, and the supernatant was removed. The trypanosomes, located in a layer on top of the sedimented red blood cells, were gently suspended in separation buffer (57 mM Na_2HPO_4, 3 mM NaH_2PO_4, 44 mM NaCl, 4 mM KCl, 5 mM glucose, 80 mM glucose, pH 8). The resuspension step was performed twice in order to ensure most of the blood cells were removed. Following the last centrifugation step the vial was tilted at a 20° angle for 5 minutes to allow the parasites to separate from the pelleted cells and settle on the side of the vial. The settled parasites were then removed from the vial and resuspended in culture medium.

3.1.5 Infection of fish

Prior to infection, blood samples from individual fish were examined for the presence of hemoflagellates. Fish were infected intraperitoneally with $6.25x10^6$ trypanosomes or an equivalent volume of control medium using a 1 mL syringe fitted with a 25-guage needle.

3.1.6 Assessment of infection (parasitemia)

To determine the number of parasites present in circulation, 50 µL samples of blood were withdrawn from the caudal vein of the fish. Samples were diluted in tri-sodium citrate anticoagulant (100 mM tri-sodium citrate, 40 mM glucose, pH 7.3) and the number of trypanosomes was determined using an improved Neubauer hemocytometer fitted with a glass cover slip (22 x 22 mm, #1 thickness), using bright field microscopy (400x). Each blood sample was enumerated by hemocytometer once since the process of counting a large number of samples was time consuming and the viability of the parasites in the anticoagulant over a long time period was uncertain.

For fish harboring small numbers of parasites that were not detectable using the hemocytometer, blood collected in a heparinized capillary tube was examined for parasites using the hematocrit centrifuge technique [401]. When no trypanosomes were detected using the hemocytometer but the number in the heparinized capillary tube was innumerable, the blood sample that had been diluted in anticoagulant was collected in a separate capillary tube, centrifuged, and re-examined.

3.1.7 Antigen preparation

3.1.7.1 Whole cell lysates

Parasites and goldfish macrophage cell lysates were prepared by washing *in vitro* cultured parasites or goldfish cells twice in culture medium (400 x *g*, 10 minutes). The parasites were then resuspended in culture medium containing protease inhibitors (1 μg/mL leupeptin, 1 μg/mL antipain, 5 μg/mL aprotinin, 1 mM phenylmethylsulfonylfluoride-PMSF) and subjected to 3 cycles of freeze-thaw.

3.1.7.2 Excretory-secretory (ES) products

Following two washes in parasite culture medium (400 x *g*, 10 minutes) *in vitro* cultured trypanosomes were adjusted to a concentration of 4.28×10^8/mL. The suspension was incubated at 20°C for 1 hour and then centrifuged (400 x *g*, 10 minutes). To ensure removal of any remaining parasites the supernatant was centrifuged at 12,000 x *g* for 10 minutes. The supernatant containing parasite ES products was stored at -20°C until needed.

3.1.7.2.1 Fractionation of trypanosome excretory-secretory products using ion exchange chromatography (Mono-Q)

Samples of parasite ES products were concentrated 20-fold using dialysis tubing (3 kDa MWCO), and polyethylene glycol flakes (Sigma) as well as centrifugal concentrators (Pall Filtron-10K molecular weight cutoff). The concentrated material was then filtered (0.22 μm syringe filter; Millipore) and dialyzed at 4°C overnight against PBS (pH 7.4) in Snakeskin dialysis tubing 3 kDa MWCO (Pierce). Ion exchange chromatography was performed using a Mono-Q column (Pharmacia) that had been pre-equilibrated with 20 mM Tris-HCl (pH 7.4) running buffer. Concentrated ES products were applied to the column at a flow rate of 0.75 mL/minute and proteins that did not bind to the column were collected in a flow-through fraction. A linear ascending salt gradient was established by running 0.5M NaCl in 20 mM Tris-HCl (pH 7.4) at a flow rate of 0.75 mL/minute through the column. Mono-Q fractions were collected in 15 mL centrifuge tubes, pooled in groups of five fractions, filter sterilized (0.22 µm syringe filter; Millipore) and stored at 4°C prior to Western blot analysis.

3.1.7.3 Detergent soluble molecules

Detergent soluble parasite extracts were prepared from *T. danilewskyi* using Triton X-114 solution as described before [40, 48]. The parasites were washed twice in serum-free medium for 10 minutes at 400 x *g* and solubilized on ice for 1 hour in membrane solubilization buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-114, 1 µg/mL leupeptin, 1 µg/mL antipain, 5 µg/mL aprotinin, 1 mM PMSF). One milliliter of parasite solution was layered onto 0.5 mL of membrane solubilization buffer containing 6% sucrose that was preheated to 30°C. After 3 minutes of incubation at 30°C the samples were centrifuged at 300 x *g* for 10 minutes and the oily droplet at the bottom of the tube containing the detergent soluble molecules was collected for use in subsequent assays.

3.1.8 Determination of packed red cell volume

Fish blood collected in a heparinized capillary tube was centrifuged for 5 minutes in a micro-hematocrit centrifuge (International Equipment Co., Needham Maryland). Red cell volume in percent was determined by comparing the ratio of red blood cells (at red/white cell interface) to whole blood (plasma/air interface) using a micro-capillary reader (Damon).

3.1.9 Anti-carp IgM antibody

Hybridoma cell supernatants containing monoclonal antibodies directed towards an epitope of the heavy chain of carp Ig (designated WCI 12) were obtained from previously grown cultures [40]. The specificity of the monoclonal antibody for goldfish immunoglobulin was determined by SDS-PAGE and Western blotting. Immunoblotting revealed a single band (approximately 80 kDa) in goldfish serum. These results corroborated the findings of Wilson *et al.* [398], who reported the heavy chain of goldfish to be ≈79 kDa.

3.1.10 Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

3.1.10.1 SDS-PAGE

Proteins were separated and visualized by reducing SDS-PAGE according to the method originally described by Laemmli [201]. Samples were dissolved in an equal volume of Laemmli sample buffer (BioRad), heated at 95°C for 5 minutes and electrophoresed through polyacrylamide gels (4% stacking, 12% separating) at 100V for 15 minutes followed by 185V for 45 minutes. In some experiments the polyacrylamide gels were stained with Coomassie brilliant blue (BioRad) or silver stained to visualize the protein bands. For Coomassie staining the gels were incubated in a solution of Coomassie brilliant blue (0.1% Coomassie brilliant blue, 10% acetic acid, 40% methanol) for 30 minutes. The gels were destained using a solution of 10% acetic acid and 40% methanol. Silver staining was performed using a silver stain kit according to manufacturer instructions (BioRad).

3.1.10.2 Western blotting

Proteins were transferred to 0.2 µm nitrocellulose membranes (BioRad) at 100V for 1 hour in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol). Membranes containing the transferred proteins were treated in the following manner. The nitrocellulose was first incubated in blocking solution consisting of 0.5% BSA in Tris-buffered saline/Tween 20 (TTBS; 0.1% Tween 20 in 100 mM Tris-HCl, 0.9% NaCl, pH 7.5; TBS) for 30 minutes at room temperature. The membranes were then immersed in a solution containing the primary antibody dissolved in blocking solution and incubated overnight at 4°C. Membranes were washed 3 times in TTBS and 3 times in TBS for at least 5 minutes each prior to reaction with secondary antibody diluted in blocking solution for 1 hour at room temperature. Following incubation with the secondary antibody, the blots were washed 3 times in TTBS and 3 times in TBS for at least 5 minutes. Protein bands were visualized using the chromogenic BCIP/NBT development kit according to the manufacturer instructions (BioRad).

3.1.11 Estimation of protein concentration

The Pierce Micro BCA protein assay was used for the determination of total protein concentration in test samples. Briefly, serial dilutions of a known concentration of bovine serum albumin (BSA) were used to create a standard curve. Unknown samples were diluted in the same buffer as the standard. Following the addition of the working reagent, the samples were mixed and heated at 60°C for 1 hour. Two hundred microlitres of each sample were added to the wells of a 96-well plate and read in an automated plate reader set at 570nm.

3.1.12 Production of polyclonal rabbit-anti-*T. danilewskyi* α-tubulin and rabbit-anti-goldfish complement component three (C3) antibodies

Purified recombinant α-tubulin was used to immunize a rabbit for production of polyclonal-antiserum. Primary injection of the rabbits was performed using 750 μL of purified recombinant α-tubulin mixed with 750 μL of Freund's complete adjuvant (FCA). Booster injections were performed every 4 weeks for 8 weeks using the same quantity of recombinant α-tubulin in conjunction with Freund's incomplete adjuvant (FIA). The specificity of the antibody for the immunizing antigen was determined using Western blot analysis. A similar protocol was performed for immunization of rabbits with goldfish C3 purified from fish serum.

3.1.13 Isolation of polyclonal IgG from rabbit serum

Polyclonal antibodies (anti-goldfish C3 and anti-recombinant α-tubulin) were purified from rabbit serum using ammonium sulfate precipitation and a HiTrap Protein A HP affinity column (Amersham Pharmacia) according to the manufacturer protocol. Briefly, 10 mL of polyclonal rabbit anti-recombinant α-tubulin serum was precipitated through the addition of 45% cold saturated ammonium sulfate. Serum was allowed to precipitate overnight at 4°C with gentle rocking. The precipitated proteins were removed by centrifugation (5000 x g; 30 minutes), resuspended in 10 mL of 20 mM sodium phosphate (pH 7) and dialyzed at 4°C overnight in 4 L of 20 mM sodium phosphate (pH 7), in 3 kDa MWCO Snakeskin dialysis tubing (Pierce). The dialyzed proteins were applied to a 1 mL HiTrap Protein A HP column that was pre-equilibrated with 20 mM sodium phosphate (pH 7). The bound protein fraction was eluted from the column with a decreasing pH gradient (0.1 M citric acid pH 3-6). The pH of the eluted fractions was neutralized by the addition of 1 M Tris-HCl pH 9. Each fraction was tested for the presence and purity of IgG using SDS-PAGE and immunblotting using a 1:3000 dilution of alkaline phosphatase (AP) conjugated goat anti-rabbit IgG. Fractions containing IgG were pooled and the protein concentration was determined using a Micro BCA protein assay reagent kit (Pierce). The purified IgG was filter-sterilized (0.22 µm syringe filter; Millipore) and stored at 4°C until used.

3.1.14 Mass spectrometry

Gel slices containing the protein bands reacting with serum from recovered goldfish were excised and sent to the UVic-Genome BC Proteomics Center located at the University of Victoria (www.proteincentre.com). Gel bands were destained, proteins were extracted and subsequently reduced (10 mM DTT), alkylated (50 mM ammonium bicarbonate), and digested in a solution of porcine modified trypsin (20 ng/µL). LC-MS/MS analysis was performed with a Hybrid Quadupole-TOF LC/MS/MS mass spectrometer (QStar Pulsar i) equipped with a nanospray ionization source. MS data was acquired automatically using Analyst QS 1.0 software Service

Pack 8 (ABI MDS SCIEX). A built in Mascot script was used to create the peak lists that were then sent to a local Mascot search engine V 2.0. The sequence database searched was MSDB, a comprehensive, non-identical protein database containing 1721490 sequences, 554556810 residues.

3.2 Isolation of *T. danilewskyi* α- and β-tubulin cDNA sequences and production of recombinant proteins

3.2.1 cDNA synthesis and RT-PCR

Total RNA was extracted from *in vitro* cultured trypanosomes using TriZol reagent according to the manufacturer instructions for cells grown in suspension (Gibco). Total RNA was extracted from $2\text{x}10^8$ parasites and RNA samples were stored at -20°C until used for RT-PCR. First-strand cDNA synthesis was performed using 5 µg of total trypanosome RNA, oligo (dT) primer, and MMLV-RT according to manufacturer instructions (Stratagene). The primer pairs used to amplify *T. danilewskyi* α and β-tubulin were ordered from Gibco (Table 3-2). The cycling parameters for the amplification of the α and β-tubulin PCR products were as follows: (1) 94°C for 1 minute 30 sec; (2) 35 cycles of 94°C for 30 sec, 61°C for 40 sec, and 72°C for 2 minutes; (3) 72°C for 12 minutes. Ten microlitres of PCR product were visualized on a 1.2% agarose gel stained with ethidium bromide (0.5 µg/mL). The PCR products generated were cloned into the pCR 2.1-TOPO vector (Invitrogen) and sequenced using the vector specific primers M13 forward and M13 reverse (Table 3-2).

3.2.2 *In silico* analysis

Sequence manipulations were performed using the Genetool (Biotools Inc., Edmonton, Canada) software package. NCBI BLAST searches were performed for sequence comparison. Sequence alignments were performed using CLUSTAL W version 1.82 [365]. Sequences for the amino acid alignments were taken from GenBank and have the following accession numbers: *T. cruzi* α-tubulin (AF455115), *T. brucei rhodesiense* α-tubulin (K02836), *Leishmania donovani* α-tubulin (U09612), *Euglena gracilis* α-tubulin (AF182309), *Onchorynchus mykiss* (Rainbow trout) α-tubulin (M36623), *Xenopus laevis* α-tubulin (X07046), *Mus musculus* α-tubulin 3

(M13442), *Homo sapiens* α-tubulin 3 (NM_006009), *T. cruzi* β-tubulin 1.9kb (AF455116), *T. brucei rhodesiense* β-tubulin (K02836), L. *mexicana* β-tubulin (AF345947), *Euglena gracilis* β-tubulin (X15797), *Notothenia coriiceps neglecta* (Antarctic yellowbelly rock cod) β-tubulin (L08013), *Xenopus laevis* β-tubulin (BC054297), *Mus musculus* β-tubulin (X04663), *Homo sapiens* β-tubulin 3 (NM_006086).

3.2.3 Cloning and expression of recombinant trypanosome tubulin subunits

T. danilewskyi α- and β-tubulin subunit gene sequences were expressed in *Escherichia coli* using the pET Directional TOPO Expression Kit (Invitrogen). PCR construct primers (Table 3-2) were designed to amplify each tubulin subunit from trypanosome cDNA according to the manufacturer's instructions. PCR parameters for amplification of the tubulin constructs were as follows: (1) 94°C for 1 minute 30 sec; (2) 35 cycles of 94°C for 30 sec, 61°C for 40 sec, and 72°C for 2 minutes; (3) 72°C for 12 minutes. Ten microlitres of PCR product were visualized on a 1.2% agarose gel stained with ethidium bromide (0.5 μg/mL). One microlitre of the PCR products was cloned into the pET/100 Directional TOPO expression vector and the plasmids were used to transform chemically competent TOP10 *E. coli* (Invitrogen) according to the manufacturer protocol. Cells were plated onto LB-ampicillin (50 μg/mL) plates and incubated overnight at 37°C. Positive clones were identified by randomly choosing 20 clones that were then used as a template for PCR reactions using the vector specific primers T7 and T7 reverse. Positive clones were grown overnight at 37°C with shaking in 2 mL of LB-ampicillin (50 μg/mL) and plasmids were prepared using a QIAprep Spin Miniprep kit (Qiagen). To verify that the expression constructs were cloned into the vector in the correct orientation, and that the constructs were in-frame, the plasmids were sequenced (with the vector-specific primers T7 and T7 reverse) using the DYEnamic ET-Terminator Cycle Sequencing Kit (Amersham Pharmacia) and Applied Biosystems 377 DNA sequencers according to standard manufacturer protocols.

Plasmid DNA containing in-frame constructs were used to transform BL21 Star (DE3) One Shot *E. coli* (Invitrogen) for recombinant protein expression. Briefly, 10 ng of plasmid DNA were transformed into the bacteria by incubation on ice followed by a short heat-shock treatment. Transformed cells were grown overnight at 37°C with shaking in LB supplemented with 50 µg/mL carbenicillin prior to the initiation of pilot expression studies.

Pilot expression studies were performed to determine the optimal incubation time for induction with IPTG according to the manufacturer protocol. Briefly, 10 mL of LB medium containing 50 µg/mL carbenicillin were inoculated with 500 µL of an overnight culture as described above and was grown for 2 hours at 37°C with shaking until the culture reached an OD_{600} of 0.5-0.8 (mid-log phase of growth). IPTG was added to a final concentration of 1 mM and a 500 µL aliquot was removed and centrifuged at 10,000 x *g* for 10 minutes in a microcentrifuge. The supernatant was removed and both the pellet and the supernatant were frozen at -20°C. These samples were considered the 0-hour time points. The remaining culture was further incubated at 37°C with shaking and 500 µL samples were removed 2, 4, and 6 hours post-induction. For each time point, the samples were processed as above and were subsequently analyzed for the presence of recombinant protein by SDS-PAGE and western blotting. For immunoblotting, the anti-HisG antibody (Invitrogen) was used at a dilution of 1:5000, the secondary antibody used was alkaline-phosphatase conjugated goat-anti-mouse IgG diluted 1:1500.

3.2.4 Scale-up expression and isolation of recombinant trypanosome tubulin subunits

For large-scale expression and purification of recombinant α- and β-tubulin, positive clones were used to inoculate 50 mL of LB medium containing 50 µg/mL carbenicillin and the cultures were grown overnight at 37°C with shaking to an OD_{600} of 1.0-2.0. Ten millilitres of the overnight culture were then used to inoculate each of four flasks containing 250 mL of LB containing 50 µg/mL carbenicillin. The cultures were incubated until mid-log phase of growth when they were induced to express the recombinant protein using 1 mM IPTG. Cultures were then grown for 4 hours prior to the purification of the recombinant tubulin sub-units.

Bacterial cultures containing the expressed proteins were centrifuged at

5000 x *g* for 15 minutes at 4°C in pre-weighed centrifuge bottles. Supernatants were removed and the wet-weight of the *E. coli* pellets was determined. Three milliliters of cold cell-lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl; pH 8.0) were added per gram of *E. coli* and the suspension was vortexed to ensure thorough mixing. For each gram of *E. coli*, 4 µL of 100 mM PMSF and 80 µL of 10 mg/mL of lysozyme were added and the suspension was rocked for 20 minutes. Next, 4 mg of deoxycholic acid was added per gram of *E. coli* and the mixture was incubated at 37°C with occasional stirring until the mixture became viscous. Following this step, 20 µL of 1 mg/mL DNAse I was added per gram of *E. coli* and the lysates were incubated at room temperature with rocking until the solution was no longer viscous and recombinant proteins were then isolated from inclusion bodies.

Isolation of the inclusion bodies was performed using a procedure adapted from Schoner *et al.*, [328]. Cell lysates from above were centrifuged at 10,000 x *g* using a microcentrifuge for 15 minutes at 4°C. The supernatants were removed and the pellet was resuspended in 1 mL of water per gram of *E. coli* and centrifuged again at 10,000 x *g* for 15 minutes. The supernatants were decanted and the pellet was re-suspended in 100 µL of Tris-HCl (pH 8.5) containing 1M urea per gram of *E. coli* (urea was added just prior to use), and centrifuged at 10,000 x *g* for 15 minutes to isolate the inclusion bodies. Sub-samples of the supernatant were retained for analysis by SDS-PAGE and the inclusion body pellet was re-suspended in 100 µL of water per gram of *E. coli* and centrifuged at 10,000 x *g* for 15 minutes. Inclusion bodies were solubilized by the addition of 100 µL of inclusion body solubilization buffer I (50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 8 M urea, and 0.1 M PMSF; pH 8) per gram *E. coli* (urea and PMSF were added just prior to use). The mixture was incubated for 1 hour at room temperature, 9 volumes of inclusion body solubilization buffer II (50 mM KH_2PO_4, 1 mM EDTA, 50 mM NaCl; pH 10.7) were added and the solution was incubated for an additional 30 minutes at room temperature. Following incubation in inclusion body solubilization buffer II, the pH of the solution was adjusted to 8 with 12 N HCl and the incubation continued for 30 minutes at room temperature. The solution was centrifuged at 10,000 x *g* for 15 minutes in a microcentrifuge and the supernatants containing the isolated recombinant proteins were collected for analysis by SDS-PAGE and Western blotting prior to renaturation. The concentration of the recombinant proteins was determined using the Micro BCA protein assay reagent kit (Pierce).

The procedure for renaturation of the proteins isolated from bacterial inclusion bodies was adapted from Hoefkins *et al.* [159]. Renaturation of proteins was performed by diluting the proteins to a concentration of 20 μg/mL in renaturation buffer (0.1 mM Na-EDTA, 0.1 mM Tris-Cl, 1 mM GSH (reduced glutathione); pH 8.2) at 4°C for 30 minutes. Oxidized glutathione (GSSG) was then added to a concentration of 0.5 mM and the solution was incubated for 22 hours at 4°C. Following incubation, the solution was dialyzed overnight against 4 L of 1X PBS pH 7.4 using 3 kDa MWCO Snakeskin dialysis membranes (Pierce). The dialyzed material was then filter sterilized (0.22 μm) and stored at 4°C prior to purification.

3.2.5 Purification of recombinant proteins

3.2.5.1 Nickel-agarose chromatography

Renatured recombinant proteins were dialyzed for 48 hrs at 4°C against 8L of NiNTA wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole; pH 8) in 3 kDa MWCO Snakeskin dialysis membranes (Pierce). The samples were then concentrated 10-fold using polyethylene glycol flakes (Sigma), dialyzed overnight at 4°C against 4 L of NiNTA wash buffer, and filtered (0.22 μm). Recombinant proteins were purified using NiNTA agarose (Qiagen). Briefly, 2 mL of settled agarose were poured into a 3 cm^3 syringe fitted with a stop-cock. The settled agarose bed was washed with 5 column volumes of NiNTA wash buffer prior to the addition of the concentrated samples containing the recombinant proteins. The liquid was allowed to flow through the column by gravity until the entire sample had passed through. The column was then allowed to stand for 1 hour at 4°C before it was washed with 5 volumes of NiNTA wash buffer. The bound recombinant proteins were then eluted with NiNTA wash buffer supplemented with 125 mM imidazole and the elutions were collected as 1 mL fractions. Samples of the wash and elution fractions were analyzed by SDS-PAGE and Western blotting. The fractions containing the purified recombinant molecules were pooled, dialyzed overnight against 4 L of PBS at 4°C, and filter sterilized (0.22 μm) prior to use.

3.2.5.2 Magnetic separation

Bacterial cell cultures expressing the recombinant tubulin subunits were prepared as described above. Following induction with IPTG, cultures were centrifuged for 15 minutes at 5000 x *g* and the cell pellets were frozen at -20°C overnight. The cell pellet was resuspended in 50 mL of MagneHis™ cell lysis reagent (diluted to 1X in wash buffer (100 mM HEPES, 10 mM imidazole, 7.5 M urea; pH 7.5) per

250 mL of original culture and incubated for 30 minutes at room temperature with agitation. Thirty microlitres of resuspended MagneHis™ Ni-particles were added per 1 mL of lysed bacteria and the suspension was incubated for 30 minutes at room temperature with mixing. To purify the recombinant proteins, the tubes containing the Ni-particles were placed in the magnetic stand for 5 minutes or until the Ni-particles were captured by the magnet. The supernatant was poured off and retained for analysis. The tube containing the Ni-particles was removed from the magnet and the Ni-particles were washed 3x with MagneHis™ wash buffer. For each wash, 5X the volume of Ni-particles was used, the particles were resuspended in the buffer, and the tube was placed in the magnetic stand until the Ni-particles were captured. The supernatant of each wash was then carefully poured off and reserved for analysis. Proteins were eluted from the Ni-particles using wash buffer containing 150 mM imidazole. One hundred microlitres of elution buffer per 30 μL of Ni-particles were used for each of 4 identical elution steps. For each elution, the particles were incubated in the elution buffer for 2 minutes at which time the tube was placed in the magnetic stand and the supernatant containing the eluted proteins was withdrawn using a pipette. Samples of the supernatant, wash steps, and elutions were analyzed by gel electrophoresis and Western blotting. Renaturation of purified recombinant molecules was performed as described previously

3.2.6 Endotoxin removal

Endotoxin (LPS) was removed from the purified, renatured recombinant proteins using a Detoxi-gel column according to the manufacturer instructions (Pierce). To ensure that the endotoxin has been removed, the solution containing the purified protein samples were analyzed using the Limulus Amoebocyte Lysate kit (Charles River Endosafe). Samples eluted from the Detoxi-gel column were found to contain <0.25 EU/mL while those samples that were not applied to the column contained ≥0.25 EU/mL.

3.2.7 Indirect immunofluorescence

In vitro-cultured trypanosomes were washed twice in serum-free medium and re-suspended to a concentration of 1x10^{6}/mL. One hundred microlitres of parasites were spun onto poly-L-lyseine coated glass slides using a Cytospin (55 x *g*, for 7 min). The parasites were fixed to the slides in methanol at -20°C for 30 minutes and permeabilized using 1% Triton X-100 for 30 minutes at room temperature. Following permeabilization, the slides were washed 3 times in Milli-Q water for 5 min each. The slides were then blocked with 0.5% BSA in PBS for 30 minutes at 37°C. For labeling of *T. danilewskyi* tubulin, slides were incubated in a 100-fold dilution of rabbit-anti-recombinant α-tubulin in PBS overnight at 37°C. The following day, the slides were washed 3 times for 30 minutes each in Milli-Q water and then blocked with 0.5% BSA in PBS for 30 minutes at 37°C. Finally, the slides were incubated in a solution of FITC-conjugated goat anti-rabbit IgG (diluted 1:100) (Pierce) for 2 hours at 37°C. The stained parasites were observed using differential interference contrast (DIC) imaging with a Leica confocal microscope.

For visualization of rabbit-anti-recombinant α-tubulin IgG that was internalized by the trypanosomes, 1x10^{6} parasites were incubated in the presence of 1X PBS, or in 1X PBS containing different concentrations of rabbit IgG, in a 96-well plate for 48 hours. The parasites were then washed twice in 1X PBS (750 x *g* for 10 minutes) and spun onto poly-L-lysiene coated glass slides as described above. The slides were fixed for 30 minutes in ice-cold methanol, and incubated with FITC-labeled goat-anti-rabbit IgG diluted 1:100 in 1X PBS for 2 hours at 37°C. Following

3 washes in Milli-Q water (20 minutes each), the stained parasites were observed using differential interference contrast (DIC) imaging with a Leica confocal microscope.

3.3 Effects of Rabbit and Goldfish Antibodies on Trypanosome Growth *In Vitro* and Immunization of Goldfish with Recombinant α- and β-tubulin Subunits

3.3.1 Determining the effects of rabbit-anti-recombinant α-tubulin polyclonal antibody on trypanosome growth *in vitro*

In vitro-cultured trypanosomes were washed twice in serum-free medium and re-suspended to a concentration of $1\mathrm{x}10^6$/mL. One hundred microlitres of parasites were added in triplicate to the wells of a 96-well cell culture plate containing 100 µL of culture medium with 10% heat-inactivated goldfish serum, or 100 µL of complete culture medium containing varying concentrations of purified rabbit-anti-recombinant α-tubulin IgG. As an additional control, washed parasites were added in triplicate to wells containing 100 µL of complete culture medium plus 100 µL containing 80 µg of rabbit-anti-goldfish C3 IgG that was also purified from rabbit serum in the manner described above. The plates were incubated at 20°C and 10 µL samples were withdrawn from each well for each of seven days. The number of parasites remaining in culture was enumerated using a hemocytometer. Each well was counted once.

3.3.2 Purification of goldfish serum IgM

IgM was partially purified from the pooled serum of healthy goldfish as described previously [415]. The goldfish serum was diluted 1:10 in 1X PBS (pH 7.4) and polyethyleneglycol (PEG) 6000 powder was added to a final concentration of 9% in small quantities with mixing over 30 minutes at room temperature. The solution was then centrifuged at 4000 x *g* for 10 minutes. The supernatant was discarded, the pellet was washed twice with a 9% PEG solution, and the pellet was subsequently redissolved in 1X PBS (pH 7.4) to the original volume of the serum. Samples of the supernatant and resuspended pellet were analyzed for the presence of goldfish IgM by immunoblotting.

For some experiments, IgM from naïve goldfish was purified further using gel permeation fast performance liquid chromatography. The PEG 6000 precipitated fraction containing partially purified IgM was filtered (0.22 μm), applied to a superose 6 column, and eluted in 1X PBS (pH 7.4) at a flow rate of 0.4 mL/minute. The fractions were collected in 15 mL centrifuge tubes and were analyzed for the presence and purity of goldfish IgM by Western blotting, and silver staining of electrophoresed samples.

3.3.3 Determining the effects of goldfish IgM on the growth of *T. danilewskyi in vitro*

PEG precipitated IgM was purified from the pooled serum of fish that had been infected with 6.25×10^6 each of *in vitro* cultured parasites for 0, 21, and 42 days. The serum from 5 fish was pooled from each day and the sera were PEG precipitated as describe above. *In vitro* cultured trypanosomes were washed twice in serum-free medium and resuspended to a concentration of 1×10^6/mL. One hundred microlitres of parasites were added in duplicate to the wells of a 96-well cell culture plate containing 100 μL of 1X PBS, 1X PBS that had been submitted to the PEG precipitation procedure, or 100 μL of 1X PBS containing various concentrations of PEG precipitated goldfish IgM. In addition, some wells contained 100 μL of PBS with different concentrations of superose-6 purified goldfish IgM isolated from the pooled serum of uninfected hosts.

For some experiments, purified goldfish IgM was pre-absorbed with 1×10^5 *in vitro* cultivated trypanosomes for 4 days in 96 well culture plates. The plates were centrifuged at 400 x *g* for 10 minute, and 100 μL of supernatant were removed and assayed for the ability of the pre-absorbed, purified IgM to inhibit parasite growth *in vitro* as described above. All plates were incubated at 20°C and 10 μL samples were withdrawn from each well on each day for 4 days. The number of parasites remaining for each treatment was enumerated using a hemocytometer. Each well was counted once.

3.3.4 Immunization of goldfish

Fish were administered 20 or 40 μg of detoxified recombinant α- and β-tubulin in conjunction with an equal volume of Freund's complete adjuvant (FCA). The immunization was performed intraperitoneally using a 25-gauge needle and a syringe. Control fish received an equivalent volume of 1X PBS in conjunction with FCA. To ensure the infectivity of the inoculum, and to control for the effects of weekly bleeding/handling, additional controls consisted of not-immunized/infected fish and not-immunized/not-infected fish.

3.4 Effects of Goldfish Complement on *T. danilewskyi In Vitro*

3.4.1 Enzymes and inhibitors

Trypsin (TPCK-treated), soybean trypsin inhibitor (SBTI), sialidases (*Clostridium perfringens, Arthrobacter ureafaciens*), and puromycin were obtained from Sigma. Trypsin and SBTI were diluted to 1 mg/mL in serum-free culture medium and used at a concentration of 0.1 mg/mL. *C. perfringens* sialidase was re-suspended to 1 U/mL in serum-free culture medium and used at 0.1 U/mL while *A. ureafaciens* α-2→(3,6,8,9)-sialidase was used at a final concentration of 0.02 U/mL. Puromycin was diluted in culture medium and used at a final concentration of 10 μg/mL.

3.4.2 Enzymatic treatment of trypanosomes

In vitro cultured trypanosomes were washed twice in serum-free medium and re-suspended to 1×10^6/mL. Enzyme solutions or medium alone were added to 1 mL of trypanosome suspension and incubated for 30 minutes at either 37°C (sialidases), or 30 minutes at 20°C (trypsin). Following enzyme treatment, 10% HI-GFS and SBTI (trypsin treatment) was added and the reaction continued for 10 minutes at either 20°C or 37°C. After inactivation of the enzymes, the parasites were washed twice (750 x *g*, 10 minutes) in serum-free medium and re-suspended to 1 mL for use in subsequent assays.

3.4.3 Assay for trypanosome lysis

To determine the susceptibility of treated and control trypanosomes to goldfish complement, 100 μL of parasites were added to 100 μL of normal or heat-inactivated non-immune normal goldfish serum, or to 75 μL heat-inactivated immune serum plus 25 μL normal non-immune serum (as a source of complement) in duplicate wells of a 96-well culture plate (Costar). For 4°C treatments, all reagents including sera were kept cold on ice. Plates were then incubated at either 4°C or 20°C for 1 hour when the contents of each well were re-suspended and the number of viable trypanosomes remaining was enumerated using a hemocytometer. Each well was counted once. The parasites were considered viable whenever flagellar movement was observed.

3.4.4 Chelation and re-supplementation of divalent cations

Prior to the addition of trypsin-treated or normal trypanosomes, 0.01 M EDTA (ethylenediaminetetraacetic acid) or, EGTA/Mg (ethylenebis (oxyethylenenitrilo) tetraacetic acid+$MgCl_2$) was added to normal or heat inactivated goldfish serum. In some cases, treated serum was re-supplemented with $MgCl_2$(0.08 M) or $CaCl_2$ (0.024 M).

3.4.5 Restoration of resistance

To determine whether treated trypanosomes could regain resistance to lysis by goldfish serum, trypsin treated or control parasites were incubated under normal culture conditions (medium+10% HI-GFS) in the presence or absence of puromycin (10 μg/mL). After 2, 4, 6, and 24 hours of incubation, 100 μL of trypanosomes were withdrawn and subjected to the assay for trypanosome lysis described above.

3.5 Attempted Identification of a *T. danilewskyi* Complement-Binding Molecule

3.5.1 Purification of goldfish complement component three (C3):

Goldfish complement component three was purified in a manner similar to the way in which C3 was purified from rainbow trout and carp [255, 263].

3.5.1.1 Rabbit-anti-carp C3 immunoglobulin

A rabbit-anti-carp C3 IgG was obtained from Dr. Miki Nakao, Laboratory of Marine Biochemistry, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Japan. The immunoglobulin was found to cross react with C3 in goldfish serum (Figure 3-1) and therefore was used to identify goldfish C3 in the following purification scheme.

3.5.1.2 Polyethyleneglycol (PEG) precipitation of goldfish serum

Blood was collected from healthy goldfish and the serum fraction was isolated in the manner described above. The serum was subsequently adjusted to 10 mM EDTA and 1 mM PMSF. Next, a volume of 15% polyethyleneglycol (PEG) 3500 equivalent to half the original volume of serum was added slowly with vortexing and the mixture was incubated for 30 minutes at room temperature. The solution was then centrifuged at 5000 x *g* for 15 minutes to collect the precipitated proteins. The pellet was resuspended in 1 mL of resuspension buffer (10 mM sodium phosphate pH 7.5, 50 mM 6-aminocaproic acid (EACA), 2 mM EDTA, 0.2 mM PMSF) and reserved for analysis by SDS-PAGE and immunoblotting. Next, a volume of 26% PEG 3500 equivalent to 75% of the original volume of serum was added to the supernatant while mixing. The solution was incubated for 30 minutes at room temperature, centrifuged at 7000 x *g* for 20 minutes, and the pellet was resuspended in 9 mL of resuspension buffer. Samples of the supernatant and pellets from each precipitation step were analyzed by gel electrophoresis and Western blotting.

3.5.1.3 Anion exchange using Q-sepharose chromatography

The resuspended pellets from the 15% and 26% PEG precipitation steps were combined and applied to a 1 mL Q-sepharose column (HiTrap Q-FF, Amersham Biosciences) according to the manufacturer instructions. Briefly, the column was washed with 5 volumes of start buffer (resuspension buffer from above) and 5 volumes of regeneration buffer (start buffer+1M NaCl). The column was then equilibrated with 10 volumes of start buffer prior to the application of the resuspended PEG precipitate. All washes and equilibration steps were performed by applying the buffer with a syringe at a flow rate of 1 mL/minute. Two millilitres of the combined pellet were applied to the column using a syringe at a flow rate of 1 mL/minute. Following the application of the sample, the bound proteins were eluted with a stepwise gradient of increasing NaCl concentration (50 mM-300 mM). Three column volumes were collected for each concentration of NaCl elution buffer. All of the elution fractions were dialyzed overnight against 4 L of 1X PBS (pH 7.2) at 4°C in Snakeskin dialysis tubing MWCO 3500 (Pierce). The column was regenerated with 5 volumes of regeneration buffer and washed with 10 volumes of start buffer. Samples of the wash step following application of the sample and the elution fractions were analyzed by SDS-PAGE and Western blotting.

3.5.1.4 Gel permeation fast performance liquid chromatography (GP-FPLC)

The proteins that were eluted in the 100 mM-250 mM fractions were pooled and separated according to size using a superose 6 column (Pharmacia). The samples were filtered (0.22 μm) prior to injection of 500 μL samples onto the column. Proteins were eluted in running buffer (resuspension buffer) at 2.5 minute intervals at a flow rate of 0.4 mL/minute, and collected in 15 mL centrifuge tubes. The fractions were stored at 4°C until they could be analyzed by gel electrophoresis and immunoblotting. Fractions from multiple column runs containing goldfish C3 were pooled and concentrated using Snakeskin dialysis tubing MWCO 3500 and polyethylene glycol flakes.

3.5.1.5 Anion exchange using Mono-Q chromatography

Ion exchange chromatography of the concentrated GP-FPLC fractions containing goldfish C3 was performed using a Mono-Q column (Pharmacia) that had been pre-equilibrated with resuspension buffer. The concentrated material was applied in multiple injections to the column at a flow rate of 0.75 mL/minute and proteins that did not bind to the column were collected in a flow-through fraction. A linear ascending salt gradient was established by running 0.5 M NaCl in resuspension buffer at a flow rate of 0.75 mL/minute through the column. Mono-Q fractions were collected in 15 mL centrifuge tubes, filter sterilized (0.22 μm syringe filter; Millipore), and incubated at 4°C prior to Western blot analysis. The fractions containing the purified goldfish C3 were pooled and concentrated using Snakeskin dialysis tubing MWCO 3500 and polyethylene glycol flakes.

3.5.2 Affinity Chromatography using goldfish C3

Purified goldfish C3 (2 mg) was subjected to limited proteolysis to generate C3a and C3b as described previously [268]. Briefly, the goldfish C3 was treated with 20 μg/mL trypsin for 30 minutes at 37°C. Soybean trypsin inhibitor (40 μg/mL) was added and the reaction was continued for another 15 minutes. The cleavage products were subsequently covalently bound to an AminoLink Plus column (Pierce) according to the manufacturer instructions. Samples of the goldfish C3 pre- and post-coupling were analyzed using Western blotting to ensure coupling of the C3 to the column. Affinity purification of trypanosome C3b-binding proteins was performed according to the column manufacturer protocol. Twenty millilitres of *T. danilewskyi* whole cell lysates equivalent to $2x10^7$/mL were applied to the column in 2 mL aliquots. Following each application, the column was incubated at room temperature for 1 hour. Following the final application, the column was washed with 1X PBS and eleven 1 mL elution fractions were collected by applying 100 mM glycine (pH 2.5). The fractions were neutralized using 1 M Tris pH 7.5. Samples of the wash and elution fractions were analyzed by gel electrophoresis and silver staining.

3.5.3 Far-Western blotting

Parasite products were separated by polyacrylamide gel electrophoresis under reducing, non-reducing, and native conditions. For native gels, the percentage of acrylamide in the separating and stacking gels was lowered to 8% and 3% respectively. The electrophoresed proteins were transferred to nitrocellulose as described above (Western blotting section). Purified goldfish C3 was cleaved by trypsin treatment (20 µg/mL) for 30 minutes at 37°C. The nitrocellulose was blocked for 30 minutes at room temperature in a solution of 0.5% BSA in Tris-buffered saline/Tween 20 (TTBS; 0.1% Tween 20 in 100 mM Tris-HCl, 0.9% NaCl, pH 7.5; TBS). Zero, 50, 100, and 250 µg of cleaved C3 in blocking buffer were then incubated with each blot for 3 hours at room temperature. Following incubation with the cleaved C3, the nitrocellulose was washed 3 times (10 minutes each) in TTBS followed by three 10 minute washes with TBS. Subsequently, the nitrocellulose was reacted with affinity purified rabbit-anti-goldfish C3 IgG diluted 1:500 in blocking buffer for 3 hours at room temperature. Prior to the addition of the enzyme-labeled antibody, 3 washes with TTBS and 3 washes with TBS were performed for 10 minutes each. Finally, the blots were incubated with alkaline phosphatase-conjugated goat-anti-rabbit IgG diluted 1:3000 in blocking buffer for 1 hour at room temperature. The final washes were performed as before, 3 washes with TTBS for 10 minutes each followed by 3 washes with TBS for 10 min each. Protein bands were visualized using the chromogenic BCIP/NBT development kit according to the manufacturer's instructions (BioRad).

3.5.4 Construction of a *T. danilewskyi* cDNA library

A cDNA library was constructed from *in vitro* cultivated *T. danilewskyi*. Eleven micrograms of mRNA were isolated from the parasites using Trizol reagent (Gibco) and the MACS magnetic mRNA isolation kit for cells and tissue (Miltenyi Biotec) according to the manufacturer instructions. The phage library was constructed by ligation of trypanosome cDNA into lambda ZAP bacteriophage using a cDNA synthesis kit, ZAP cDNA synthesis kit, and the ZAP-cDNA Gigapack III Gold cloning kit (Stratagene). The titre of the unamplified library was determined to be 4000 pfu/mL with an average insert size of 1740bp. The library was subsequently amplified ($1x10^{10}$ pfu/mL) according to the manufacturer protocol and aliquots were stored at 4°C and -80°C.

3.5.5 Screening of the trypanosome cDNA library using a *T. danilewskyi* sialidase domain probe

PCR amplification of the sialidase domain of *T. danilewskyi trans*-sialidase was performed using *T. danilewskyi* cDNA (obtained as described above) as a template and the following PCR parameters: (1) 94°C for 1 minute 30 sec; (2) 35 cycles of 94°C for 30 sec, 60°C for 40 sec, and 72°C for 2 minutes; (3) 72°C for 12 minutes. Ten microlitres of the PCR product were electrophoresed in an agarose gel and stained using ethidium bromide. The PCR product was sequenced as describe previously to confirm its identity and the product was labeled using the Random Primers DNA Labeling System (Invitrogen). The radiolabeled probe was purified using gel purification columns (Qiagen). The cDNA library screening procedure was performed by plating the amplified cDNA on NZY agar plates, and plaque lifts were performed using nylon membranes. Membranes were prehybridized for 5 hours at 42°C in prehybridization solution (15.3 mL Milli-Q water, 15 mL formamide, 12.5 mL 20xSSC (sodium chloride-sodium citrate), 0.25 mL 20% SDS (sodium dodecyl sulfate), 5 mL 50X Denhardt's solution, 2 mL calf thymus DNA) prior to the addition of the sialidase probe. The lifts were hybridized with the probe overnight at 42°C. Following hybridization, the membranes were washed 3 times for 5 minutes in 2xSSC, 0.1% SDS and 3 times for 20 minutes in 0.1xSSC, 0.1% SDS. After washing, the membranes were exposed to film and stored overnight at -80°C. Putative positive plaques were identified following development of the film, and the isolated plaques were stored at 4°C in 500 μL of SM buffer + chloroform for analysis by PCR. Sequencing of the putative positive clones was performed using T3 and T7 primers (Table 3-2) using the DYEnamic ET-Terminator Cycle Sequencing Kit (Amersham Pharmacia) and Applied Biosystems 377 DNA sequencers according to standard manufacturer protocols.

3.6 Statistics

For the effects of immunoglobulins on the growth of parasites *in vitro*, one-way analysis of variance was used to calculate differences between treatment groups. For immunization experiments, a one way repeated measures ANOVA was performed to compare differences in parasitemia between immunization treatments. Logarithmic transformation of the data was performed to stabilize the variances; 1 was added to each value prior to transformation. All analyses were performed using Super Anova software for the Power Macintosh. The probability level of $P \leq 0.05$ was considered significant.

For complement studies, statistical analyses were performed using SYSTAT software (Systat Software, Inc., Point Richmond, CA, USA). Factorial analysis of variance was used to test for treatment effects on trypanosome counts (transformed by $\log_{10} x+1$). When significant treatment effects were detected, multiple comparisons were done to identify specific factor levels responsible for the effect. These comparisons used the Bonferroni adjustment [343] to maintain an experiment-wise error rate $P \leq 0.05$.

Table 3-1. Composition of *T. danilewskyi* culture medium[a]

Reagent/Solution	Amount/L	Specifications
Milli-Q water	300 mL	Milli-Q PF Plus System
Hank's solution	40 mL	10X, no Ca^{2+} and Mg^{2+}
MEM amino acid solution[b]	12.5 mL	50X
MEM non-essential amino[b] acid solution	12.5 mL	100X
$NaHCO_3$	1.26 g	
NaOH	0.15 mL	1N
Sodium pyruvate solution[b]	12.5 mL	10 mM
MEM Vitamin solution[b]	10 mL	100X
Nucleic acid precursor[c] solution	10 mL	2.5 mM
L-glutamine solution	10 mL	200 mM
Gentamicin solution[b]	1 mL	50 mg/mL
2-mercaptoethanol solution	1 mL	50 mM
HEPES	2 g	
Insulin	0.01 g	
GFL-15 medium[d]	500 mL	

[a] Developed by Wang and Belosevic [390]

[b] Purchased from Gibco/Invitrogen

[c] 2.5 mM each of adenosine, cytidine, hypoxanthine, thymidine, and uridine

[d] Leibovitz-15 and Dulbecco's Modified Eagles Medium 50:50 (v/v)

Table 3-2. List of primers[a]

Primers		5'-3'
T. danilewskyi α-tubulin	F	CACCCGTGAGGCGATTTGCATCCAC
	R	GCCCTTTGCGCCTAGTAATTCCTC
T. danilewskyi β-tubulin	F	CACCATGCGTGAGATTGTGTGCGTT
	R	TCCTTGCGTCCGCTCAGTACT
TOPO TA (M13)	F	GTAAAACGACGGCCA
	R	CAGGAAACAGCTATGAC
PET directional TOPO T7	F	TAATACGACTCACTATAGGG
	R	TAGTTATTGCTCAGCGGTGG
T. danilewskyi sialidase domain	F	CGGCGGAAATGGAAGGAATGC
	R	CACCCAGCAAAGGACTCGC
T. cruzi complement regulatory protein (CRP)	F1	CATGGCACCCCGCACAC
	R1	CGCCGACGTTCTCACCC
	F2	AGCGGAAGGAGGGAAGGA
	R2	TGGCGGAATGGGACGACGAC
T3 λ phage-specific primer		ATTAACCCTCACTAAAGGGA
T7 λ phage-specific primer		TAATACGACTCACTATAGGG

[a] Primers purchased from Invitrogen/Gibco

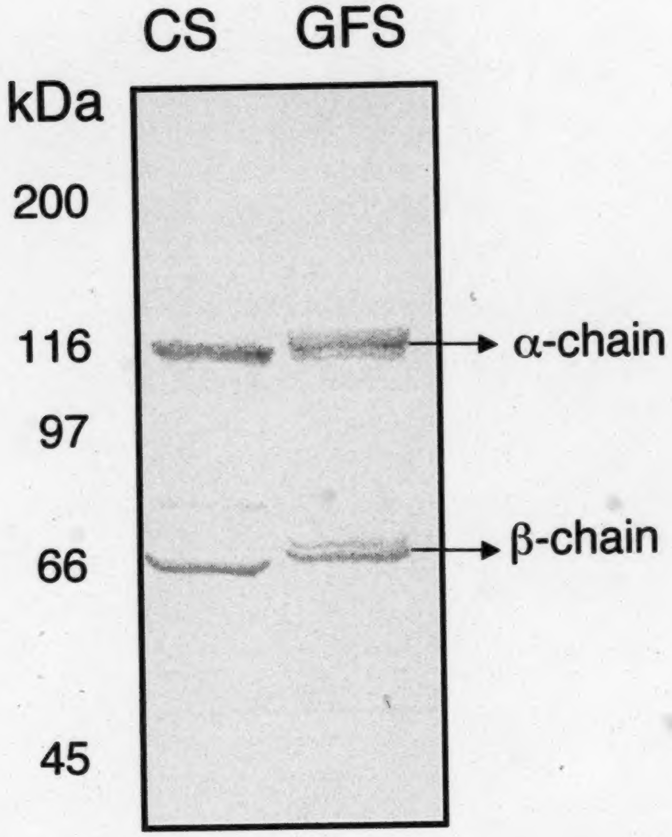

Figure 3-1. Purified rabbit-anti-carp complement component three (C3) IgG cross-reacts with C3 in goldfish serum. One microlitre of carp (CS) and goldfish (GFS) serum were separated using SDS-PAGE and the proteins were transferred to nitrocellulose. The membrane was incubated with the rabbit-anti-carp C3 IgG diluted 1:1000 in blocking buffer and subsequently reacted with alkaline phosphatase labeled goat-anti-rabbit IgG. Following addition of the enzyme substrate two bands corresponding to the α (115 kDa) and β (75 kDa) chains (arrows) of C3 were identified in both the carp and goldfish sera.

CHAPTER 4

DESCRIPTION OF THE COURSE OF INFECTION

4.1 Introduction

A number of species of fresh water fish are susceptible to infection by *Trypanosoma danilewskyi* including the common carp (*Cyprinus carpio*) from which it was first described in 1904. The parasites have a wide host range, being naturally infective to tench (*Tinca, tinca*) and eel (*Anguilla* spp.) in Europe and Asia [217], and experimentally infective to numerous other species including tin foil barb (*Barbus schwanenfeldi*), brown bullhead (*Ictalurus nebulosus*), and goldfish (*Carassius auratus*) [215, 405]. The parasites are transmitted by blood feeding leeches of the genera *Hemiclepsis* and *Pisciola* [300, 405]. The parasites develop in the stomach of the leech and subsequently migrate to the crop where metacyclic trypanosomes are inoculated into a new host during the next blood meal. In the laboratory, parasites isolated from infected fish can be injected into the peritoneal cavity of goldfish to establish a new infection. Alternatively, the parasites can be cultivated *in vitro* in culture medium containing fish serum and then used to initiate an infection in the goldfish [41, 390]. Cultures of *T. danilewskyi* consist primarily of bloodstream (trypomastigote) forms of the parasites (Figure 4-1) [40]. The purpose of the studies described below was to characterize the growth of *T. danilewskyi* during *in vitro* cultivation and during the course of infection in goldfish (*Carassius auratus*).

4.2 Experimental Design

The growth of *in vitro* cultivated parasites under standard maintenance conditions was assessed by inoculating 25cm^2 tissue culture flasks with 1x10^5 trypanosomes/mL of culture medium. Subsequently, the number of parasites in each flask was determined every other day for 8 days. Parasites were enumerated by performing counts of two diluted samples from each flask using a hemocytometer.

The generation time (g) of the parasites was determined using the following formula [390]:

$$g = t_2\text{-}t_1/\mathrm{log}e^{n2/n1}$$

where t_1 = time of inoculation; t_2 = end time point; n1 = number of parasites in the inoculum; n2 = number of parasites at the end point.

For analysis of the course of infection *in vivo*, goldfish measuring 9.5-11cm in standard body length were inoculated intraperitoneally with $6.25\text{x}10^6$ *in vitro* cultured parasites. Uninfected control fish were inoculated with an equal volume of serum-free parasite culture medium. The number of parasites/mL of blood was determined on the third day post-infection, and subsequently at weekly intervals, using a hemocytometer or the hematocrit centrifuge technique, when numbers of parasites were below the detection limit of the hemocytometer [401].

4.3 Results

Infection of naïve fish with *in vitro* cultured *T. danilewskyi* resulted in 100% prevalence of infection after 7 days (Figure 4-2 B). None of the control fish developed a hemoflagellate infection nor was any mortality observed in this group. The course of infection involved an early rise in parasitemia in which parasite infection was detected in 90% of goldfish after only 3 days. Parasite numbers continued to rise, culminating in a peak of infection after 14 days followed by a gradual decline in the mean number of parasites in the blood. The majority of the fish (90%) remained infected after 56 days (Figure 4-2 A). Cumulative mortality after 8 weeks of infection was low, only 1 fish out of 10 died (Figure 4-2 C). The packed red cell volume of infected and uninfected fish decreased similarly over the sampling period from 0 to 28 days post infection and subsequently rebounded to near starting levels on day 56 (Figure 4-2 D).

The trypanosomes were found to proliferate *in vitro* in the presence of 10% goldfish serum. Based on the average number of parasites from the three culture flasks on day 8, the generation time of the parasites was calculated to be 47.4 hours and there was a greater than 9-fold increase in parasite concentration over the course of the experiment (Figure 4-3).

4.4 Discussion

The goldfish used in this study were highly susceptible to infection with *T. danilewskyi*. However, despite the high dose of parasites used in the inoculum, very little mortality was observed. The *in vitro* cultured trypanosomes were highly infective and most of the fish developed infections as early as three days following infection. The course of infection is consistent with what has been previously observed for *T. danilewskyi* infections in goldfish and it is expected that most of the fish would maintain low level chronic infections if the experiment had continued past 56 days [40, 389]. The mortality that is associated with *T. danilewskyi* infections in goldfish has previously been shown to be dependent on the number of parasites in the initial inoculum [390, 403]. Mortality of 31% and 75% was observed in fish that were infected with five hundred thousand and two million parasites respectively [390]. In another study, 18% mortality wasdocumented for fish that were infected with more than 6 million parasites [40]. The results of my experiments are similar to those of the latter investigation in which 10 naïve fish were infected with 6.25×10^6 parasites and 90% of fish survived the infection.

There are numerous possible explanations for the decrease in mortality associated with *T. danilewskyi* infection when fish are injected with *in vitro* cultivated parasites. The first is the source of the infecting parasites since more *in vitro* cultured parasites than blood-derived trypanosomes were required to achieve the same mortality [403]. It is certainly possible that the *in vitro* cultivated parasites have lost virulence and infectivity. However, it has been shown that *T. danilewskyi* grown in cultures containing 5% carp serum and 5% fetal bovine serum are indistinguishable from parasites isolated from infected fish in terms of surface structure, monoclonal antibody reactivity, and morphology [284]. In addition, the fact that the course of infection initiated by cultured parasites *in vivo* has remained the same for more than 10 years does not suggest that the parasites have become less infective or more susceptible to fish immune defenses. Morphometric studies of the life cycle forms of parasites grown in culture have shown that the majority of parasites found in culture represent bloodstream forms and that the proportion of these decreases in culture from 80% to 60% after one week [40]. Because the parasites used for infection are normally isolated from cultures that are 1 week old, a lower concentration of parasite life cycle stages adapted to living in the fish may result in an attenuated infection. A second explanation for the decreased mortality of infected fish might be related to the variability in the immune responses of outbred fish populations, in addition to the susceptibility of the fish immune response to different environmental factors such as temperature and water quality. It has been well established that fish that have been exposed to hemoflagellate infections and recover are immune to subsequent reinfection. Because the fish received from the suppliers are not certified pathogen-free, some individuals may have protective immunity that is the result of a heterologous hemoflagellate infection.

Anemia is one symptom that has been associated with *T. danilewskyi* infections in fish and has been previously shown to correlate with high numbers of circulating parasites in the blood [168, 389]. The results of this study support the observations of other researchers [40] suggesting that anemia associated with experimental infections in goldfish may be a result of repeated anesthesia and blood sampling, rather than a direct result of high parasite loads. Packed red cell volumes were measured throughout the experiment as an indication of anemia and although there was a decrease in percent red cell volume observed over the first 28 days of infection, there was a concomitant decrease in red cell volume in control fish. The average red cell volume of each group of fish was found to have returned to pre-infection levels when fish were sampled 4 weeks later. Alternatively, it is possible that the parasites have lost the ability to induce the anemia that was previously observed. Mechanisms by which the parasites might induce anemia include production of enzymes that directly lyse red blood cells, or induction of polyclonal antibody production in which auto-reactive or cross-reactive antibodies might bind to host erythrocytes and induce lysis.

Serum from different species of fish including carp, tinfoil barb, and goldfish, potentiate the growth of *T. danilewskyi* in culture. Alternatively, medium containing mammalian serum does not support the growth of the trypanosomes [41]. The generation time of the parasites cultivated *in vitro* in 10% goldfish serum (47.4 hours) was similar to the generation time that was previously reported for parasite growth under the same conditions (44.4 hours) [390]. Therefore, the characteristics of the growth of *T. danilewskyi* in 10% goldfish serum reported in this study support previous findings and indicate that the capacity of the parasites to grow in culture has not changed in at least 8 years. The ability to cultivate parasites *in vitro* is advantageous since large numbers of parasites can be generated in a short time. In fact the results of my studies suggest that the same number of parasites can be obtained in culture a week sooner than the achievement of peak parasitemia in infected goldfish.

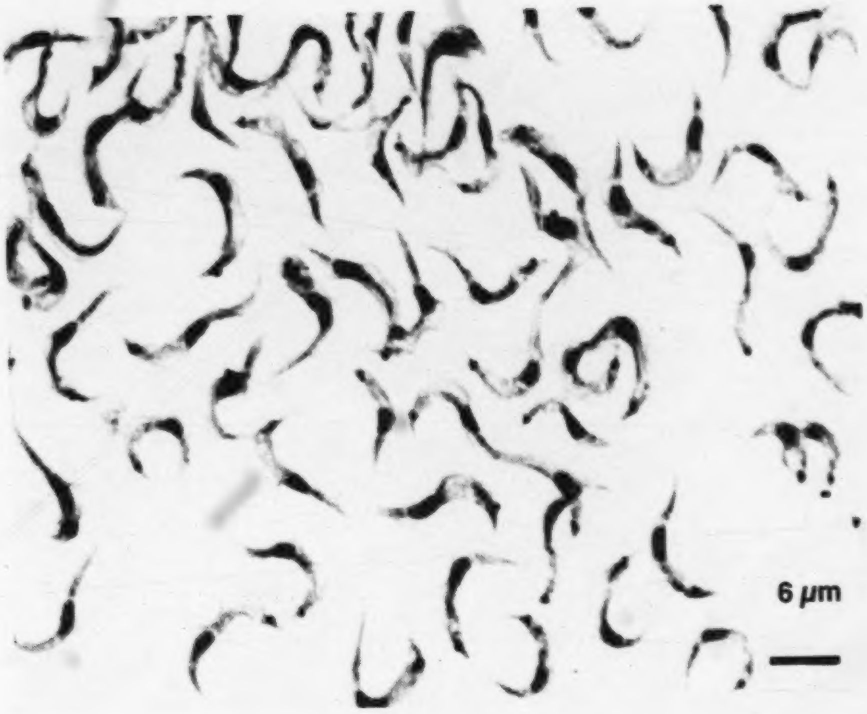

Figure 4-1. Wright's-stained smear of *Trypanosoma danilewskyi* grown for 6 days in culture medium supplemented with 10% goldfish serum. Adapted from Bienek, 2001 [40].

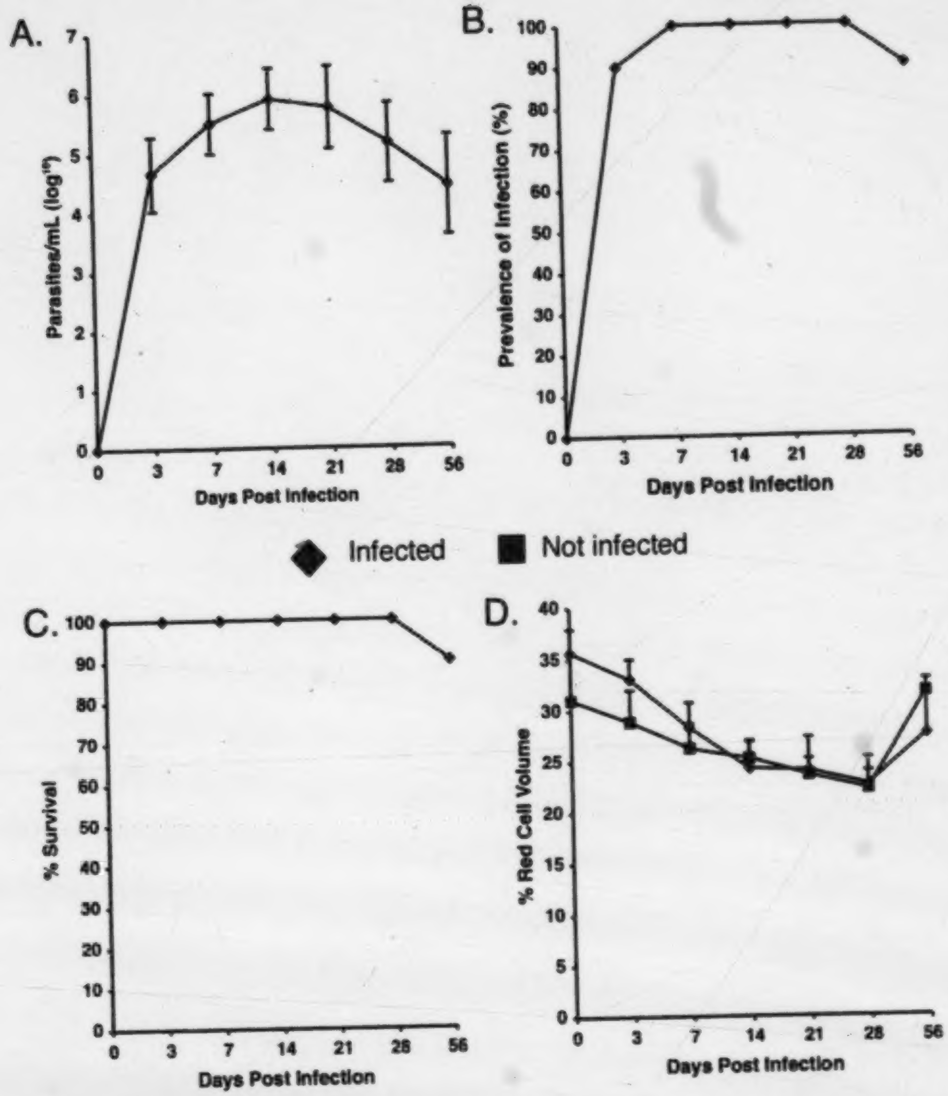

Figure 4-2. Course of infection (A), prevalence (B), mortality (C), and volume of red blood cells (D) in naïve goldfish (*n*=10) inoculated with 6.25x10^6 *in vitro* cultivated *Trypanosoma danilewskyi*. In (A), and (D) points represent the mean number of parasites/mL and mean packed red cell volume ±SEM.

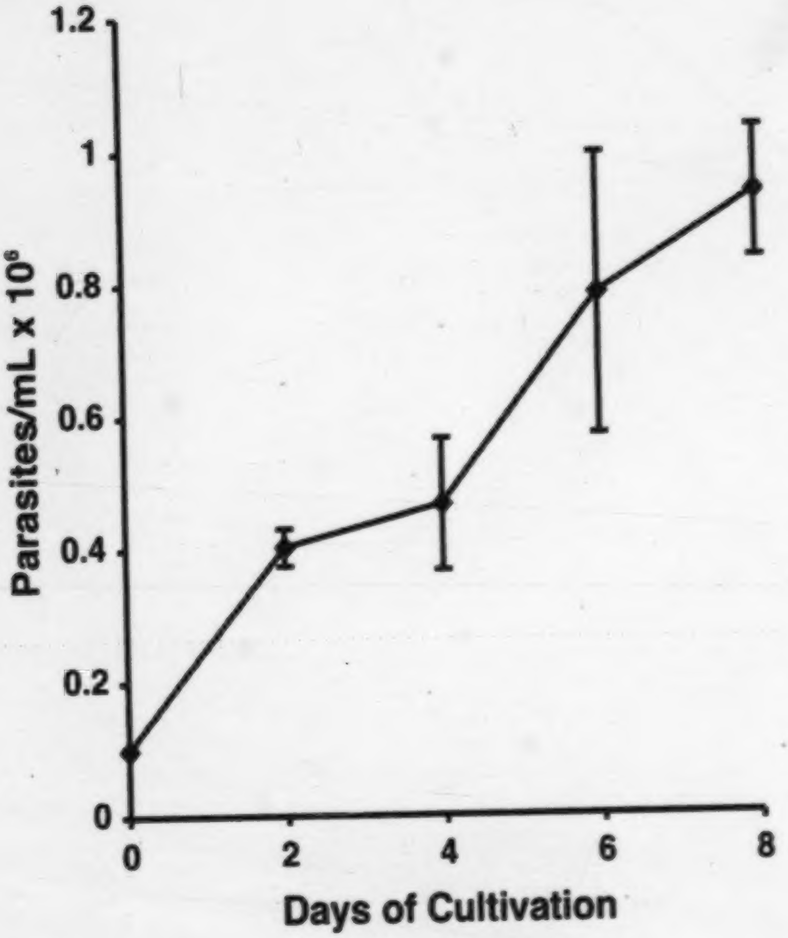

Figure 4-3. *In vitro* growth of *Trypanosoma danilewskyi* in medium supplemented with 10% heat-inactivated goldfish serum (56°C for 30 minutes). Parasites were seeded into 25 cm^2 culture flasks (n=3) at a concentration of approximately 1×10^5/mL. Each point represents the mean number of trypanosomes/mL of medium ±SEM.

CHAPTER 5

IDENTIFICATION OF *T. danilewskyi* TUBULIN IN PARASITE EXCRETORY-SECRETORY PRODUCTS AND EXPRESSION OF RECOMBINANT PARASITE TUBULIN SUBUNITS

5.1 Introduction

Immunity of fish to *T. danilewskyi* is considered to be antibody mediated for two reasons. The first reason is because experimentally infectioned goldfish that have recovered from an infection are subsequently immune to re-infection. The immunity of the fish is thought to be non-sterile and lasts for at least 190 days post-infection [390]. Secondly, passive transfer studies have shown that injection of serum, or purified IgM, from recovered fish can confer protection to naïve hosts [283, 403]. The identity of the parasite molecules responsible for inducing the production of protective antibodies in the fish are of great interest because they represent potential candidates for treatment of, or vaccination against, *T. danilewskyi* infections in fish.

We have previously demonstrated [43] that immunization of goldfish with excretory/secretory (ES) products of *T. danilewskyi* results in increased resistance to challenge infections. Specifically, those fish immunized with ES products had a lower prevalence of infection than fish that had been administered control medium alone. Fish immunized with ES products in conjunction with Freund's complete adjuvant (FCA) were found to harbor 5, 4, and 2 logarithms fewer parasites than controls on days 7, 14, and 28 post-infection, respectively [43].

The objectives of the studies described in this chapter were: (1) to identify the component(s) of *T. danilewskyi* ES products that induce the production of specific antibodies in infected goldfish; and (2) to identify the cDNA sequences encoding the recognized protein(s) for production of recombinant molecules in a prokaryotic expression system. Recombinant proteins were expressed in order to generate material for production of antibodies to *T. danilewskyi* antigens and for use in goldfish immunization experiments (Chapter 6).

5.2 Experimental Design

5.2.1 Identification of the antigenic component(s) of *T. danilewskyi* excretory-secretory products

To identify antigenic molecules that were excreted or secreted by the parasites, crude excretory-secretory (ES) products were analyzed in a number of ways including size exclusion chromatography, concanavalin-A affinity chromatography, and 2-dimensional SDS-PAGE. The presence of antibodies to ES products was determined by Western blotting with pooled serum samples from naïve fish, and from fish that had recovered from *T. danilewskyi* infection. A procedure was optimized involving anion exchange chromatography to achieve fractionation of ES products, and Western blotting using serum from fish that had been infected with *T. danilewskyi* for 56 days. Coomassie stained gel slices containing protein bands of interest were excised and sent to the UVic-Genome BC Proteomics Center located on the University of Victoria campus (www.proteincentre.com). Results of Q-TOF-Nanospray-tandem mass spectrometry (PE SCIEX API Pulsar), and peptide mapping of trypsin-digested samples were compared to a database of known proteins to identify the fractionated protein.

5.2.2 Amplification of *T. danilewskyi* α- and β-tubulin gene sequences, cloning, and expression of recombinant proteins

The cDNA molecules encoding the parasite proteins were amplified using RT-PCR and the full-length coding sequences of both α- and β-tubulin were determined. Full-length PCR products were generated using construct primers that enabled the gene sequences to be cloned in-frame into a prokaryotic expression vector for production of recombinant *T. danilewskyi* α- and β-tubulin.

The vectors encoding the α- and β-tubulin subunits were used to transfect *Escherichia coli* that could be induced to produce recombinant α- and β-tubulin in milligram quantities in a short amount of time. The recombinant proteins were expressed with a 6Xhistidine sequence at the N-terminus of the molecule that facilitated their identification in bacterial cell lysates and subsequent purification steps. The 6Xhis tag was used to purify the protein using nickel affinity chromatography or, by magnetic separation utilizing nickel particles. The purified and re-natured α-tubulin was then used to immunize a rabbit for production of a polyclonal antibody. IgG was purified from the rabbit-anti-recombinant α-tubulin anti-serum using Protein A affinity chromatography. The purified rabbit-anti-recombinant α-tubulin IgG was used to identify the native tubulin molecule in trypanosome whole cell lysates, and it was also used in immunofluorescence studies to label permeabilized parasites isolated from the blood of infected fish. In order to determine whether *T. danilewskyi* internalize the purified rabbit-anti-recombinant α-tubulin IgG, the parasites were incubated with different concentrations of the rabbit antibody, washed, fixed to glass slides, and stained with FITC-labeled goat-anti-rabbit IgG.

5.3 Results

5.3.1 Identification of tubulin in *T. danilewskyi* ES products

Western blotting using fractions from Mono-Q fractionated and non-fractionated parasite ES products with serum from uninfected (non-immune) and recovered goldfish (immune, 56 days post-infection) revealed a band of approximately 52 kDa that reacted with pooled sera from recovered fish (Figure 5-1). The band was subsequently determined by mass spectrometric analysis to contain peptides having sequence similarity to *Trypanosoma cruzi* α- and β-tubulin. In order to determine the role of these molecules in the immune response of goldfish to the parasites, the cDNAs encoding the *T. danilewskyi* α- and β-tubulin were cloned and expressed using a prokaryotic expression system.

5.3.2 Identification of *T. danilewskyi* α- and β-tubulin cDNA sequences

The cDNA encoding *T. danilewskyi* α-tubulin was determined to be 1650 nucleotides and the coding sequence consists of 1353 base pairs corresponding to 451 amino acids (Figure 5-2). The coding region of the nucleotide sequence shares 99% identity with the α-tubulin sequences from *T. cruzi*, 98% with *T. brucei rhodesiense*, and slightly less with *L. donovani* (93%). Acetylation of α-tubulin on lysine 40 is a common post-translational modification associated with stable microtubules (such as those found in the sub-pellicular lattice) and this residue was conserved among all of the species examined including *T. danilewskyi* α-tubulin [143] (Figure 5-3). The cDNA encoding the *T. danilewskyi* β-tubulin was found to be 1541 nucleotides and the coding region consists of 1326 base pairs corresponding to 442 amino acids (Figure 5-4). The coding region of the β-tubulin nucleotide sequence shares 98% identity with *T. cruzi*, 97% with *T. brucei rhodesiense*, and 95% with *L. mexicana*. The sequence of *T. danilewskyi* β-tubulin revealed that the first four amino acids of the predicted protein sequence correspond to MREI, which are conserved residues of β-tubulin proteins from other organisms [28] (Fig 5-5). The asparagine residue at position 100 that has been associated with cell types exhibiting sensitivity to rhizoxin was also predicted to be conserved in the *T. danilewskyi* β-tubulin amino acid sequence [143]. One characteristic of both α and β-tubulin among the trypanosomes is the specification of a tyrosine residue as the C-terminal amino acid that can be removed by a carboxypeptidase after the tubulin is incorporated into a microtubule. As a result, older microtubules contain mainly detyrosinated tubulin [408]. Both the α-tubulin and β-tubulin sequences from fish trypanosomes have a predicted tyrosine residue at the C-terminus of the protein sequence indicating that they may be susceptible to similar post-translational modifications (Figure 5-3 and 5-5). The *T. danilewskyi* tubulin sequences have been submitted to the GenBank database (α-tubulin accession no. DQ080027; β-tubulin accession no. DQ080028).

5.3.3 Cloning of *T. danilewskyi* α- and β-tubulin into pET100/D-TOPO vector expression, and purification of recombinant proteins

Using the prokaryotic expression construct primers (Table 3-2), PCR products were generated that corresponded to the expected sizes of *T. danilewskyi* α- and β-tubulin (1365bp and 1326bp respectively) (Figure 5-6). Each PCR product was cloned into the pET100/D-TOPO vector. Maximal induction of protein expression in BL21 Star (DE3) *E. coli* with 1 mM IPTG was determined to be between 2 and 4 hrs for both α-tubulin (Figure 5-7 A), and β-tubulin (Figure 5-7 C). Both of the expressed recombinant proteins corresponded to a size of 55-60 kDa. The expression of recombinant α- and β-tubulin subunits resulted in the compartmentalization of the proteins into insoluble bacterial inclusion bodies. Consequently, the molecules were purified from inclusion bodies and renatured as described in the materials and methods (Chapter 3). The recombinant α-tubulin possessing the 6xHis tag was successfully recovered from the renatured material using a nickel-agarose column and the bound proteins were eluted with imidazole (Figure 5-7 B). Although the same procedure was performed for the purification of recombinant β-tubulin, an alternate purification protocol was also developed in which nickel beads were added to lysed *E. coli* to bind 6XHis tagged protein. The nickel beads with the bound recombinant proteins were subsequently recovered from the lysates using magnetic separation. The bound recombinant protein was eluted from the nickel beads using imidazole (for detailed procedures see materials and methods). The proteins eluted from the beads were renatured using the protocol previously established for renaturation of proteins isolated from inclusion bodies. Since the procedure involving the magnetic separation of proteins resulted in a greater yield of purified recombinant α- and β-tubulin in fewer steps, this procedure was chosen for all subsequent purifications (Figure 5-7 D).

5.3.4 Recognition of native and recombinant α- and β-tubulin by polyclonal rabbit anti-serum

Rabbit-anti-recombinant α-tubulin antiserum was generated toward purified recombinant α-tubulin and the IgG fraction was isolated using affinity chromatography. Using Western blotting, it was determined that the purified IgG fraction of the antiserum recognized recombinant α- and β-tubulin (Figure 5-8 A2 and B2), as well as the native *T. danilewskyi* tubulin present in whole cell lysates (Figure 5-8 A1). There was no reactivity of the anti-parasite-α-tubulin IgG with goldfish macrophage lysates (Figure 5-8 B1). The apparent difference in size of the recombinant molecules as compared to the native molecules is due to the additional weight added from the vector sequence.

5.3.5 Indirect immunofluorescence

Permeabilized trypanosomes were uniformly stained with rabbit anti-recombinant α-tubulin IgG (Figure 5-9 A). No fluorescence was observed when slides were incubated with the isotype control (Figure 5-9 B). Note that the antibody staining in A did not apply to the goldfish blood cell that was also captured in the photo indicating that the antibody is specific for *T. danilewskyi* tubulin.

Internalized anti-recombinant α-tubulin antibody was also detected inside of trypanosomes that were exposed to 80 and 160 μg of IgG for 2 days (Figure 5-10 B, centre and right panel respectively). No fluorescence was detected when parasites were incubated with PBS for the same time period (Figure 5-10 A, left panel). When the trypanosomes were incubated with 80 μg of IgG, specific intracellular structures that might represent the nucleus or a lysosomal compartment were predominantly stained. However, when the parasites were exposed to 160 μg of IgG for the same time period, the entire parasite was brightly labeled.

5.4 Discussion

In this study the results of the identification of *T. danilewskyi* tubulin as a component of the excretory/secretory products of the parasites that induce a protective immune response in goldfish are presented. Previously, we have shown that the ES products of *T. danilewskyi* are capable of inducing protective immunity in goldfish when administered in conjunction with Freund's complete adjuvant [43]. Subsequently, fractionation of the ES products, Western blotting, and mass spectrometry were used to identify trypanosome tubulin as a component of the ES mixture that induced an antibody response in infected fish.

Tubulin molecules are cytoskeletal proteins that polymerize to form microtubules. Each tubulin molecule is a heterodimer consisting of an α subunit and a β subunit. Microtubules are of particular importance in trypanosome biology since they are critical components of the subpellicular lattice, a network of microtubules that helps to maintain the shape and structure of the organism, and they are also important for motility (flagellar axoneme), and cell division (mitotic spindle) [143]. The results of a number of studies involving mammalian trypanosomatids have identified tubulin as an immunogen despite its relatively high degree of sequence conservation among different species, and its intracellular location. For example, parasite tubulin has been identified in the urine of patients infected with *T. cruzi*, suggesting that tubulin can be found in the extracellular environment. The mechanism by which this occurs is unknown; however, it can be speculated that the tubulin could be released into circulation from parasites lysed during the course of the infection [36]. Other intracellular molecules from *T. cruzi* induce antibody production in host cells, including mitochondrial malate dehydrogenase [250], and unmethylated CpG motifs [54]. β-tubulin is an important T-cell stimulating antigen during *Leishmania* infection and anti-tubulin antibodies are present in the sera of dogs infected with *L. donovani* [287, 299]. Most recently, it has been shown that immunization of mice with tubulin purified from *T. brucei* can protect animals from homologous challenge [220]. In addition, antibodies prepared against purified native tubulin and β-tubulin C-terminal synthetic peptides prevent the growth of *T. brucei* in culture. These results suggest a possible mechanism of action for protective antibodies induced in immunized animals [221].

There are a number of possible explanations for the presence of tubulin in *T. danilewskyi* excretory/secretory products. The first is that death of the parasites occurs during the incubation of parasites, or during the centrifugation steps that are required to remove whole parasites from the preparation. In this case, tubulin would not represent a true excretory/secretory product; however, the results of these experiments suggest that it is able to stimulate antibody production in goldfish. Another possible explanation is the production of molecules by the parasites that are tubulin-like, or that contain epitopes that cross-react with parasite tubulin such as those that have been identified for *T. cruzi* [8, 9]. Antigenic mimicry is a common strategy employed by parasites, including trypanosomes, to evade host immune defenses [412]. In fact mimicry of host molecules has also been hypothesized to be associated with the autoimmune-type pathology induced by *T. cruzi* during the chronic stages of the infection [361].

In vivo, *T. danilewskyi* tubulin could be released into circulation following lysis of a proportion of the inoculated parasites. It has been demonstrated that different life cycle stages and forms of *T. brucei* and *T. cruzi* are particularly adapted to exist within their respective intermediate or definitive hosts. The parasites accomplish this feat by developmentally regulating the expression of molecules required for resistance to the immune defenses of the invertebrate and vertebrate hosts. For example, the molecules that are found on the surface of insect-stages of *T. brucei* allow them to resist invertebrate antimicrobial compounds and proteases; however, these molecules confer no protection from the vertebrate host defenses [392]. Alternatively, the infective forms of the parasites, and the stages that are found in the blood, possess other developmentally regulated proteins that confer resistance to the immune mechanisms employed by vertebrates. Parasites that are inoculated into the host that do not possess the factors that will permit immediate survival will succumb to the innate immune defenses of the vertebrate host. For example, *T. cruzi* epimastigotes from the vector do not possess the surface complement regulatory protein found in the trypomastigote stage. As a result, they are susceptible to complement-mediated lysis leading to the release of trypanosome intracellular contents [147, 335].

It has previously been shown that the majority of the parasite forms found in a flask of *in vitro* cultured *T. danilewskyi* are bloodstream forms; however, there is a small proportion of other parasite stages including stumpy, and dividing forms [40]. The differential susceptibility of the various trypanosome culture forms to goldfish immune defenses is unknown. Similarly, nothing is known about the differences between the life cycle stages found in the intermediate host and the forms found in the fish. Like the *T. cruzi* epimastigotes, some of the non-trypomastigote forms of the parasites may have an increased susceptibility to lysis by complement and, as a result, intracellular products such as tubulin could be released into the extracellular environment to stimulate an acquired immune response.

Anti-tubulin antibodies have been found in the sera of a number of species of vertebrates that have never been infected with, or immunized against, trypanosomes [188]. The presence of these natural antibodies to tubulin has also been reported in different species of fish [133]. The role of the anti-tubulin antibodies in mammalian hosts has not been determined; however, the antibodies are normally present in low concentrations that may increase following infection with a number of different parasites. Examples include *Leishmania* and *T. cruzi* and immunization with *T. cruzi* exoantigens enhances anti-tubulin antibody titers [141, 165, 185]. In many cases, the antibodies react with both host and parasite tubulin and in *T. cruzi* infections these autoantibodies are believed to play a role in the destruction of host tissue during chronic Chagas' disease. Interestingly, the pre-existing anti-tubulin antibodies and the infection-induced antibodies do not recognize common epitopes nor do they possess the same affinities for their respective binding sites [364]. The naturally occurring antibodies recognize sequences in the central portion of the molecule that may be important in regulating nucleotide binding and microtubule polymerization. On the other hand, the induced antibodies recognize epitopes at the N and C-termini with high affinity. These could also prevent microtubule polymerization in an unregulated manner [234]. Since polyreactive, natural antibodies are thought to be important in the immune response of fish [51] it is possible that anti-tubulin antibodies are present in goldfish in low concentrations that are subsequently enhanced during trypanosome infection, or following immunization with parasite extracts.

To examine the role of *T. danilewskyi* tubulin as an immunostimulatory molecule in goldfish, we cloned and expressed α-tubulin and β-tubulin using a prokaryotic expression system. The sequences that have been determined for *T. danilewskyi* α-tubulin and β-tubulin share considerable similarity to the deduced sequences for related parasite species. Both sequences retain a number of conserved residues that are commonly associated with post-translational modifications and binding properties of other tubulin molecules [143].

The genes encoding the sub-units were expressed in *E. coli* since the rapid generation time of the bacteria facilitates the production of large quantities of protein in a short time, and since tubulin from other trypanosome species has been successfully expressed in bacteria [408]. Using the renatured recombinant molecules for our studies is advantageous since we are able to avoid the potentially confounding effects of other parasite antigens that may co-purify with the native tubulin during the isolation process. A polyclonal antibody was generated towards the renatured recombinant α-tubulin molecule and the affinity-purified IgG was found to recognize recombinant α- and β-tubulin, as well as the native trypanosome tubulin molecule in parasite cell lysates. These results indicate that there are antigenic portions of the recombinant molecule that are specific to *T. danilewskyi* tubulin but that are not related to the appended vector sequence. In contrast, the rabbit anti-recombinant α-tubulin IgG did not recognize tubulin in goldfish macrophage whole cell lysates suggesting that an autoimmune response to the *T. danilewskyi* tubulin subunits used in future immunization experiments would likely not occur. The specificity of the affinity-purified anti-α-tubulin IgG was confirmed using immunofluorescence microscopy, which revealed uniform, specific, labeling of permeabilized trypanosomes.

As mentioned previously, a protective immune response was generated against *T. brucei* infection in mice immunized with purified *T. brucei* tubulin [220]. Mice were also immunized with denatured tubulin, synthetic tubulin peptides and rat tubulin; however, none of these treatments induced significant antibody production or protection from homologous challenge [220]. In order to determine the mode of activity of anti-tubulin antibodies against *T. brucei*, the parasites were incubated with different dilutions of anti-serum generated towards various tubulin preparations. The anti-tubulin anti-serum was effective in preventing the growth of *in vitro* cultivated parasites in a dose-dependent fashion. Although antibodies were also produced towards denatured tubulin and synthetic tubulin peptides, antibodies generated towards the native molecule were the most effective in preventing parasite growth [221].

Although the precise mode of action of anti-tubulin antibodies towards trypanosomes has not been determined, it is known that mammalian trypanosomes bind anti-tubulin antibodies in the flagellar pocket, and that anti-tubulin antibodies can be found inside membrane-permeabilized parasites [221]. Rabbit anti-recombinant *T. danilewskyi* α-tubulin IgG could also be detected intracellularly in non-permeabilized parasites that were pre-incubated with the antibody. The specific internal structures that were observed when the parasites were incubated with 80 μg of IgG could not be positively identified but they might represent the trypanosome nucleus or membrane-bound compartments formed as part of the endocytic pathway. These brightly staining structures could be identified using electron microscopy or by co-staining with dyes that are specific for intracellular organelles. Internal accumulation of the antibody was even more striking at higher IgG concentrations since heavy staining of whole parasites was observed. In other eukaryotic cells, monoclonal anti-tubulin antibodies inhibit motility of the flagellum, and prevent assembly of microtubules *in vitro* [91, 272]. Therefore, like *T. brucei,* internalization of anti-tubulin antibodies might play a significant role in limiting the proliferation of *T. danilewskyi in vitro* [221].

The results presented in this chapter, in addition to the reports from the literature describing mammalian model systems, suggest that parasite tubulin represents a good immunotherapeutic target for mammalian trypanosomes, and now for trypanosomes of cold-blooded lower vertebrates such as fish [221].

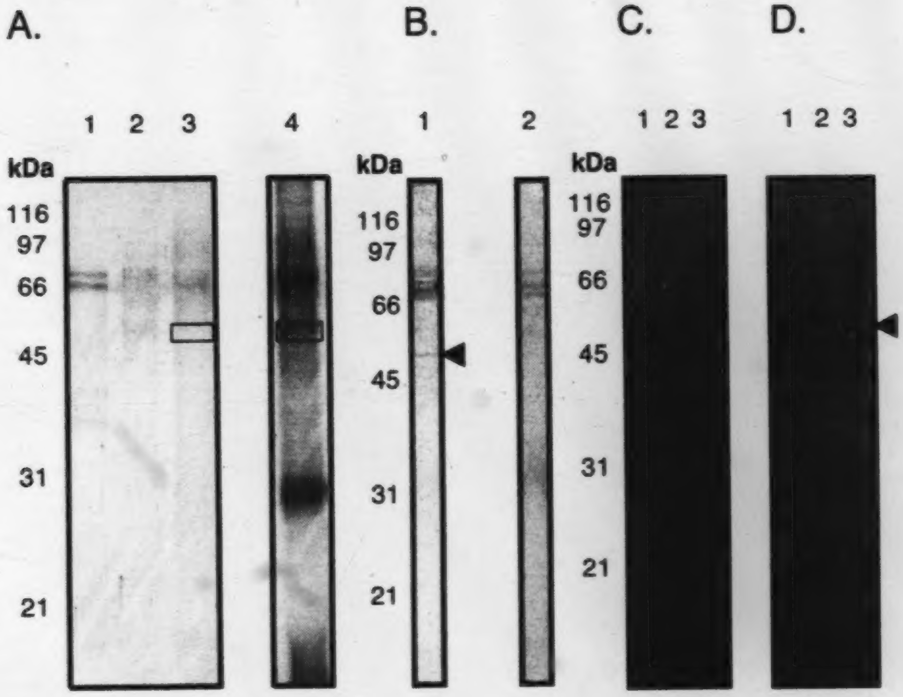

Figure 5-1. Identification of molecules contained in the excretory/secretory (ES) products of *Trypanosoma danilewskyi* that induce antibody production in infected goldfish. Crude ES products (A4 and B) as well as ES products fractionated by anion exchange chromatography and pooled (A1-3, C, and D; 1=fractions 1-5, 2=fractions 6-10, 3=fractions 11-15) were separated using SDS-PAGE and subsequently stained using Coomassie brilliant blue or transferred to nitrocellulose as described in the methods. For immunoblotting nitrocellulose was incubated with either serum from recovered goldfish 56 days post-infection (B1, D) or serum from uninfected fish (B2, C) diluted 1:25 in blocking buffer. Blots were subsequently incubated with hybridoma supernatants (diluted 1:5) from mouse containing antibodies directed towards an antigenic determinant of the heavy chain of carp IgM. Finally, the nitrocellulose was incubated with alkaline phosphatase-labeled goat-anti-mouse IgG diluted 1:1500 in blocking buffer prior to the addition of the enzyme substrate. The 52 kDa band (arrow and boxed) present in the fractionated ES products were excised from the gel and analyzed by mass spectrometry.

```
                                                              M   R   E   A   I   C
   1 ttc aaa cac cct caa ata aac tca aat cca aaa aca cca aca atg cgt gag gcg att tgc
      I   H   I   G   Q   A   G   C   Q   V   G   N   A   C   W   E   L   F   C   L
  61 atc cac att ggc cag gcc ggt tgc cag gtc ggt aat gcg tgc tgg gag ctg ttc tgc ctt
      E   H   G   I   Q   P   D   G   A   M   P   S   D   K   T   I   G   V   E   D
 121 gag cac ggc atc cag ccg gac ggc gca atg ccg tcg gac aag acg atc ggc gtg gag gac
      D   A   F   N   T   F   F   S   E   T   G   A   G   K   H   V   P   R   A   V
 181 gac gcg ttc aac acc ttc ttc tcg gag act ggc gcc ggc aag cac gtg ccg cgc gcg gtc
      F   L   D   L   E   P   T   V   V   D   E   I   R   T   G   T   Y   R   Q   L
 241 ttc ctg gac ctg gag ccg acg gtg gtg gac gag atc cgc acc ggg acg tac cgc cag ctg
      F   H   P   E   Q   L   I   S   G   K   E   D   A   A   N   N   Y   A   R   G
 301 ttc cac ccc gag cag ctg atc tcc ggc aag gag gac gca gcc aac aac tac gct cgc ggc
      H   Y   T   I   G   K   E   I   V   D   L   C   L   D   R   I   R   K   L   A
 361 cac tac acc atc ggc aag gag atc gtg gac ctg tgc ctc gac cgc atc cgc aag ctc gcg
      D   N   C   T   G   L   Q   G   F   L   V   Y   H   A   V   G   G   G   T   G
 421 gac aac tgc acc ggc ctg cag ggc ttc ctc gtg tac cac gcc gtg ggc ggc ggc aca ggc
      S   G   L   G   A   L   L   L   E   R   L   S   V   D   Y   G   K   K   S   K
 481 tcc ggc ctc ggc gcg ctg ctg ctt gag cgc ctc tcc gtg gac tac ggc aag aag tcc aag
      L   G   Y   T   V   Y   P   S   P   Q   V   S   T   A   V   V   E   P   Y   N
 541 ctc ggc tac aca gtc tac ccg tcc ccg cag gtg tcc aca gcg gtg gtg gag ccc tac aac
      S   V   L   S   T   H   S   L   L   E   H   T   D   V   A   A   M   L   D   N
 601 tct gtg ctc tcc acg cac tca ctg ctt gag cac acc gat gtt gcg gcg atg ctg gac aat
      E   A   I   Y   D   L   T   R   R   N   L   D   I   E   R   P   T   Y   T   N
 661 gag gcg att tac gat ttg acc cgt cgt aac ctc gac att gaa cgc ccg acg tac aca aac
      L   N   R   L   I   A   Q   V   V   S   S   L   T   A   S   L   R   F   D   G
 721 ctg aac cgc ctc atc gct cag gtg gtc tca tcg ctg acg gcg tcg ctc cgc ttc gac ggc
      A   L   N   V   D   L   T   E   F   Q   T   N   L   V   P   Y   P   R   I   H
 781 gcg ctg aac gtg gac ctg acg gag ttc cag acc aac ttg gtg ccg tac ccg cgc atc cac
      F   V   L   T   S   Y   A   P   V   I   S   A   E   K   A   Y   H   E   Q   L
 841 ttc gtg ctc acg agc tac gcg ccg gtg atc tca gcg gag aag gcg tac cac gag cag ctg
      S   V   S   E   I   S   N   A   V   F   E   P   A   S   M   M   T   K   C   D
 901 tcc gtg tcg gag atc tca aac gcg gtc ttc gag ccg gcg tcg atg atg acc aag tgc gac
      P   R   H   G   K   Y   M   A   C   C   L   M   Y   R   G   D   V   V   P   K
 961 ccg cgc cac ggc aag tac atg gcg tgc tgc ctc atg tac cgc ggc gac gtg gtg ccg aag
      D   V   N   A   A   V   A   T   I   K   T   K   R   T   I   Q   F   V   D   W
1021 gat gtg aac gcc gcg gtc gcg acg atc aag acg aag cgc acg atc cag ttc gtc gac tgg
      S   P   T   G   F   K   C   G   I   N   Y   Q   P   P   T   V   V   P   G   G
1081 tcg ccc act ggc ttc aag tgc ggc atc aac tac cag ccg ccc acg gtg gtg ccc ggt ggc
      D   L   A   K   V   Q   R   A   V   C   M   I   A   N   S   T   A   I   A   E
1141 gac ctg gcc aag gtg cag cgc gcc gtg tgc atg atc gcg aac tcg acg gcc atc gcc gag
      V   F   A   R   I   D   H   K   F   D   L   M   Y   S   K   R   A   F   V   H
1201 gtg ttc gcg cgc atc gac cac aag ttc gac ctt atg tac agc aag cgt gcc ttc gtc cac
      W   Y   V   G   E   G   M   E   E   G   E   F   S   E   A   R   E   D   L   A
1261 tgg tac gtc ggc gag ggt atg gag gag ggc gag ttc tcc gag gcc cgc gag gac ctc gcc
      A   L   E   K   D   Y   E   E   V   G   A   E   S   G   D   L   E   G   E   E
1321 gcg ctc gag aag gac tac gaa gaa gtc ggc gcc gaa tcc ggc gac ctc gag ggc gag gag
      D   V   E   E   Y   *
1381 gac gtg gag gaa tac tag gcg caa agg gcg aat tct gca gat att cat cac gcg acg ggc
1441 tct gtg aga gaa tcg cga cag gaa gag aga gag aga agc atg gtg tgt gtg tgg gtg tgt
1501 ggg tgc gct tcc ctg agc cgt ttc cgg ggt ttg ggg gat att ttt tat ttg acc ctt ttt
1561 ttt tac tga ttt ttt ttg ttt tct ctt cga tgt ttc ctt gat tgt gag agc gaa aaa aaa
1621 aaa aaa aaa aaa aaa aaa aa
```

Figure 5-2. The nucleotide sequence of *Trypanosoma danilewskyi* α-tubulin. The cDNA sequence for the α-tubulin gene was obtained using RT-PCR and the obtained sequence shows ~99% similarity to *T. cruzi* and *T. brucei* α-tubulin and 93% similarity to the α-tubulin sequence of *Leishmania*. The coding sequence consists of 1353bp corresponding to 451 amino acids. Initiation and termination codons are shown in bold, the lower case represents non-coding regions. This sequence has been submitted to GenBank database (accession no. **DQ089927**).

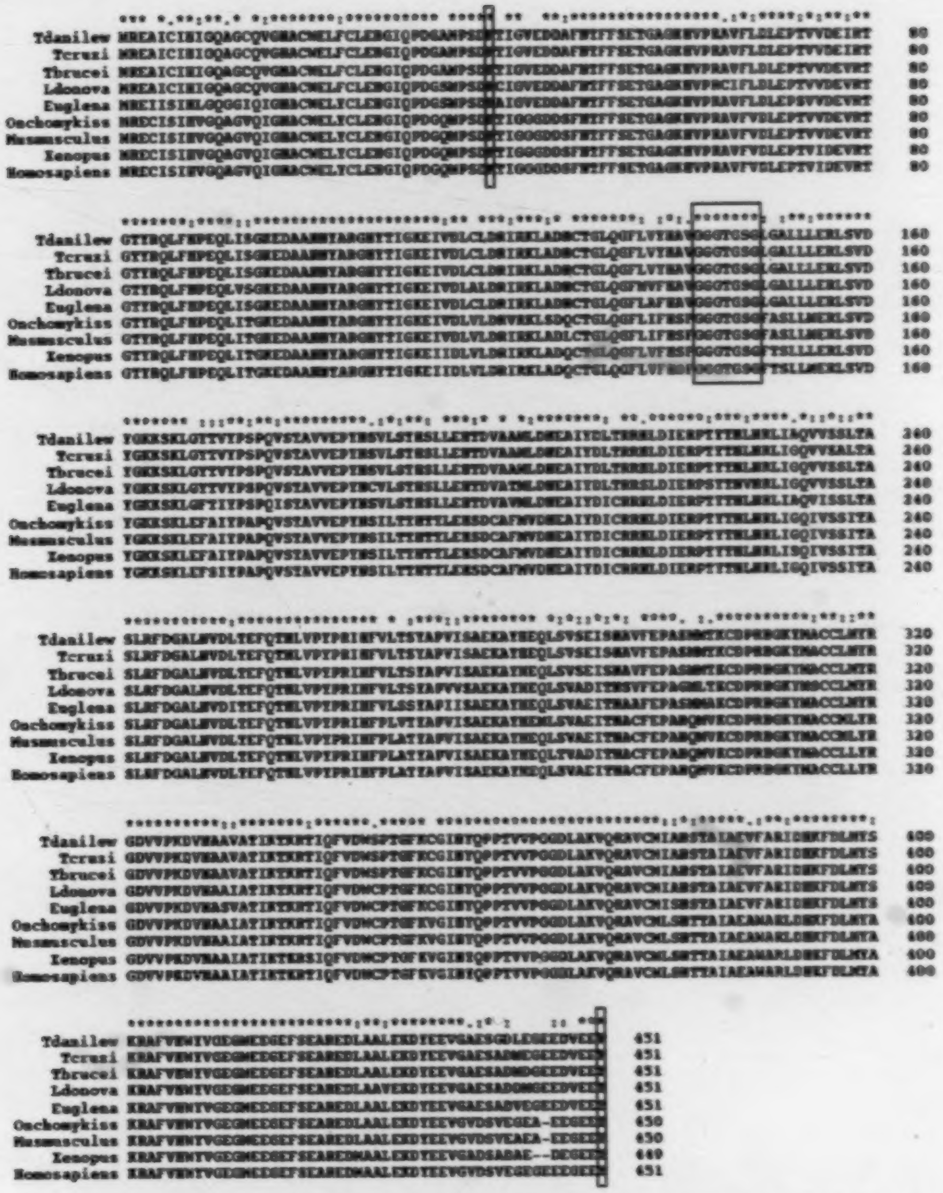
Tdanilew
Tcruzi
Tbrucei
Ldonova
Euglena
Onchomykiss
Musmusculus
Xenopus
Homosapiens
80
160
240
320
400
451
450
449

Figure 5-3. Amino acid alignment of the predicted *Trypanosoma danilewskyi* α-tubulin sequence with sequences from other euglenids (including three trypanosomatids) as well as sequences from mammalian and non-mammalian vertebrates. All sequences were obtained from GenBank; accession numbers can be found in the Materials and Methods section. '*' represents identical residues, ':' represents conserved substitutions, '.' represents semi-conserved substitutions, and '-' are gaps. Lysine residue at position 40, GTP binding site, and C-terminus tyrosine residues are conserved between all species (boxed).

```
      M   R   E   I   V   C   V   Q   A   G   Q   C   G   N   Q   I   G   S   K   F
   1 ATG cgt gag att gtg tgc gtt cag gcc ggc cag tgc ggc aac cag atc ggc tca aag ttc
      W   E   V   I   S   D   E   H   G   V   D   P   T   G   T   Y   Q   G   D   S
  61 tgg gag gtg atc agc gac gag cac ggc gtg gac ccc acc ggc acg tac cag ggc gac tcg
      D   L   Q   L   E   R   I   N   V   Y   F   D   E   A   T   G   G   R   Y   V
 121 gac ctt cag ctg gag cgc atc aac gtg tac ttt gat gag gcg acg ggc ggc cgc tac gtg
      P   R   A   V   L   I   D   L   E   P   G   T   M   D   S   V   R   A   G   P
 181 ccc cgc gcc gtg ttg atc gac ctg gag ccc ggc acg atg gac tcg gtg cgc gcc gga ccg
      Y   G   Q   I   F   R   P   D   N   F   I   F   G   Q   S   G   A   G   N   N
 241 tac ggc cag atc ttc cgc ccg gac aac ttc atc ttt ggc cag tcg ggc gcc ggc aac aac
      W   A   K   G   H   Y   T   E   G   A   E   L   I   D   S   V   L   D   V   C
 301 tgg gcc aag ggc cac tac acg gag ggc gcg gag ctc atc gac tcc gtt ctc gac gtg tgc
      R   K   E   A   E   S   C   D   C   L   Q   G   F   Q   I   A   H   S   L   G
 361 cgc aag gag gcg gag agc tgc gac tgc ctt cag ggc ttc cag att gcc cac tcg ctc ggc
      G   G   T   G   S   G   M   G   T   L   L   I   S   K   L   R   E   E   Y   P
 421 ggt ggc acg ggc tcc ggc atg ggc acc ctg ctc atc tcg aag ctg cgc gag gag tac ccc
      D   R   I   M   M   T   F   S   I   I   P   S   P   K   V   S   D   T   V   V
 481 gac cgc atc atg atg acc ttc tcc atc att ccg tcg ccc aag gtg tcc gac acg gtc gtc
      E   P   Y   N   T   T   L   S   V   H   Q   L   V   E   N   S   D   E   S   M
 541 gag ccg tac aac acg acg ctc tcc gtg cac cag ctg gtc gaa aac tct gac gaa tcg atg
      C   I   D   N   E   A   L   Y   D   I   C   F   R   T   L   K   L   T   T   P
 601 tgc atc gat aat gag gct ttg tac gat atc tgt ttt cgt act ctt aag ttg acg acg ccc
      T   F   G   D   L   N   H   L   V   S   A   V   M   S   G   V   T   C   C   L
 661 acg ttc ggc gac ttg aac cac ttg gtg tcc gct gtg atg tcc ggc gtc acg tgc tgc ctc
      R   F   P   G   Q   L   N   S   D   L   R   K   L   A   V   N   L   V   P   F
 721 cgc ttc ccc ggc cag ctg aac tcg gat ctc cgc aag ctg gcg gtg aac ctg gtg ccg ttc
      P   R   L   H   F   F   M   M   G   F   A   P   L   T   S   R   G   S   Q   Q
 781 ccc cgc ctg cac ttc ttc atg atg ggc ttc gcc ccg ctg acg agc cgc ggc tcg cag cag
      Y   R   G   L   S   V   P   E   L   T   Q   Q   M   F   D   A   K   N   M   M
 841 tac cgc ggc ctg tcg gtg ccg gag ctg acg cag cag atg ttc gat gcg aag aac atg atg
      Q   A   A   E   P   R   H   G   R   Y   L   T   A   S   A   L   F   R   G   R
 901 cag gcc gca gaa ccg cgc cac ggt cgc tac ctg acg gcg tcc gcg ctc ttc cgc ggc cgc
      M   S   T   K   E   I   D   E   Q   M   L   N   V   Q   N   K   N   S   S   Y
 961 atg tcg acc aag gag att gac gag cag atg ctc aac gtg cag aac aag aac tcg tcc tac
      F   I   E   W   I   P   N   N   I   K   S   S   I   C   D   I   P   P   K   G
1021 ttc att gag tgg atc ccg aac aac atc aag tcc tcc atc tgc gac atc ccg ccc aag ggc
      L   K   M   A   V   T   F   I   G   N   N   T   C   I   Q   E   M   F   R   R
1081 ctc aag atg gcg gtc acg ttc atc ggc aac aac act tgc att cag gag atg ttc cgt cgc
      V   G   E   Q   F   T   A   M   F   R   R   K   A   F   L   H   W   Y   T   G
1141 gtg ggc gag cag ttc act gcc atg ttc cgc cgc aag gcc ttc ttg cac tgg tac acg ggt
      E   G   M   D   E   M   E   F   T   E   A   E   S   N   M   N   D   L   V   S
1201 gag ggc atg gac gag atg gag ttc acc gag gcg gag tca aac atg aac gat ctt gtg tcc
      E   Y   Q   Q   Y   Q   D   A   T   V   E   E   E   G   E   F   D   E   E   E
1261 gag tac cag cag tac cag gac gca acg gtc gag gag gag ggt gag ttc gac gag gag gag
      Q   Y   *
1321 cag tac TAA gcg gac gca agg agg agt gta agg acg aat acc aaa gga tga agc gaa gaa
1381 gaa gag gag gag gag gag ggg aga gag aaa ttg aga gag aga gag aag aat ggg aat aga
1441 tcg gtg agg gaa agg agt gaa ggt gca caa cgg ttt ctg tcc ccg act ggg cac cat atg
1501 tgt gct cac acc aaa aaa aaa aaa aaa aaa aaa aaa aaa aa
```

Figure 5-4. The nucleotide sequence of *Trypanosoma danilewskyi* β-tubulin. The cDNA sequence for the β tubulin gene was obtained using RT-PCR and the sequence shows >90% similarity to *T. cruzi*, *T. brucei*, and *Leishmania* β-tubulin. The coding sequence consists of 1326bp corresponding to 442 amino acids. Initiation and termination codons are shown in bold, the lower case represents non-coding regions. This sequence has been submitted to GenBank database (accession no. **DQ080028**).

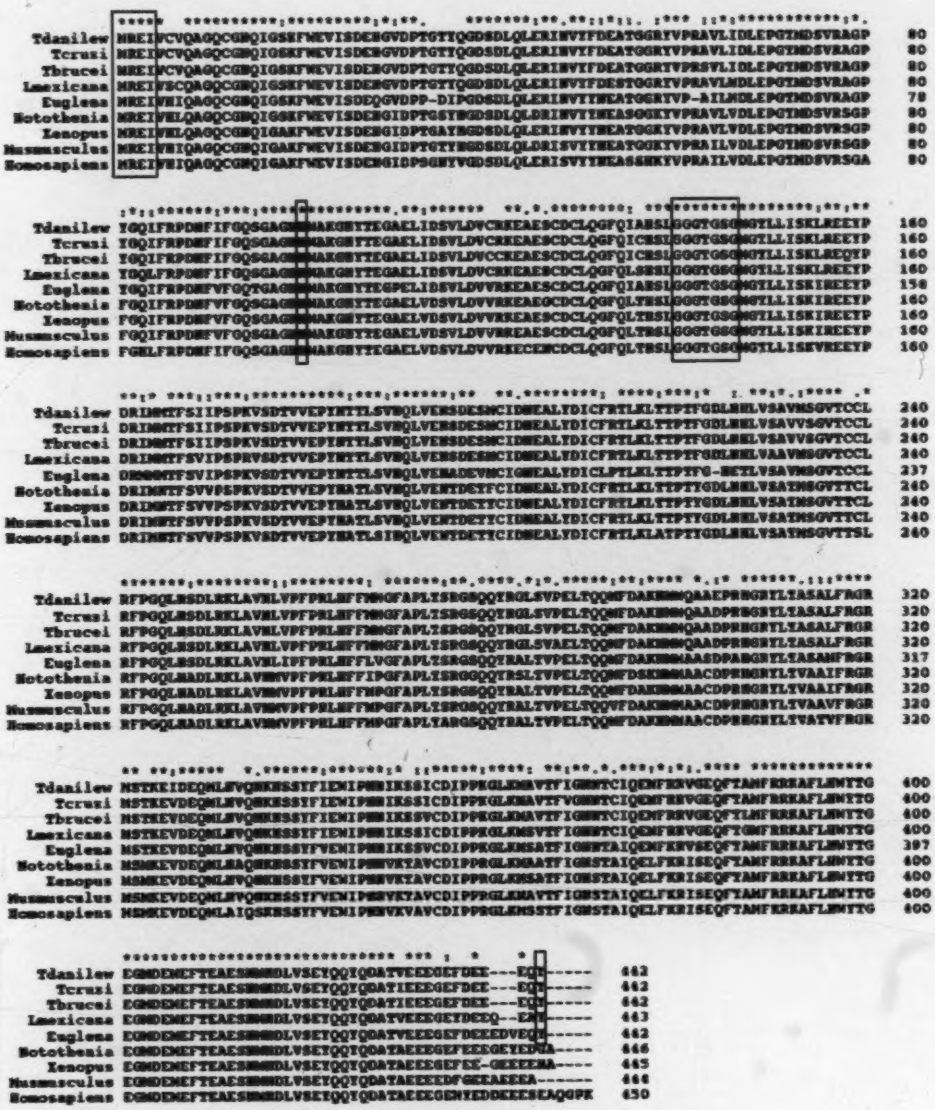
Tdanilew
Tcruzi
Tbrucei
Lmexicana
Euglena
Notothenia
Xenopus
Musmusculus
Homosapiens
80
78
160
158
240
237
320
317
400
397
442
443
446
445
444
450

Figure 5-5. Amino acid alignment of the predicted *Trypanosoma danilewskyi* β-tubulin sequence with sequences from other euglenids (including three trypanosomatids), as well as sequences from mammalian and non-mammalian vertebrates. All sequences were obtained from GenBank, accession numbers can be found in the methods section. '*' indicates identical residues, ':' represents conserved substitutions, '.' represents semi-conserved substitutions, and '-' are gaps. N-terminus MREI sequence, GTP binding site, and asparagines residue at position 100 (box) are conserved in all species. C-terminus tyrosine residue (box) is present in sequences of flagellates only.

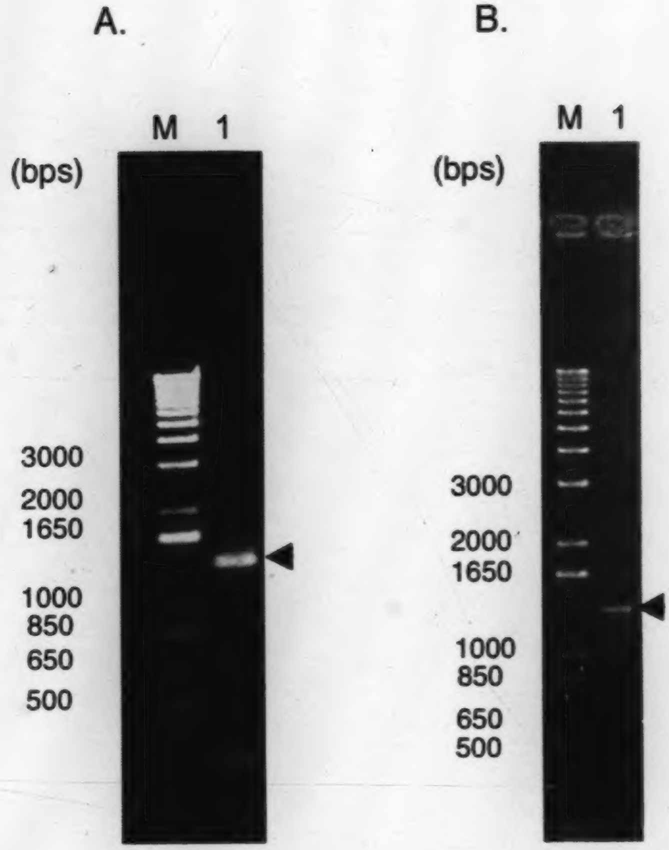
A.
M 1
(bps)
3000
2000
1650
1000
850
650
500
B.
M 1
(bps)
3000
2000
1650
1000
850
650
500

Figure 5-6. Amplification of *Trypanosoma danilewskyi* α-(A) and β-tubulin (B) gene products amplified by RT-PCR from trypanosome total RNA using construct primers for cloning into the pET100D TOPO prokaryotic expression vector. Lane M-1Kb Plus DNA ladder; lane A1, α-tubulin PCR product (arrow); lane B1, β-tubulin PCR product (arrow). PCR products were separated on a 1.2% agarose gel and visualized by staining with ethidium bromide.

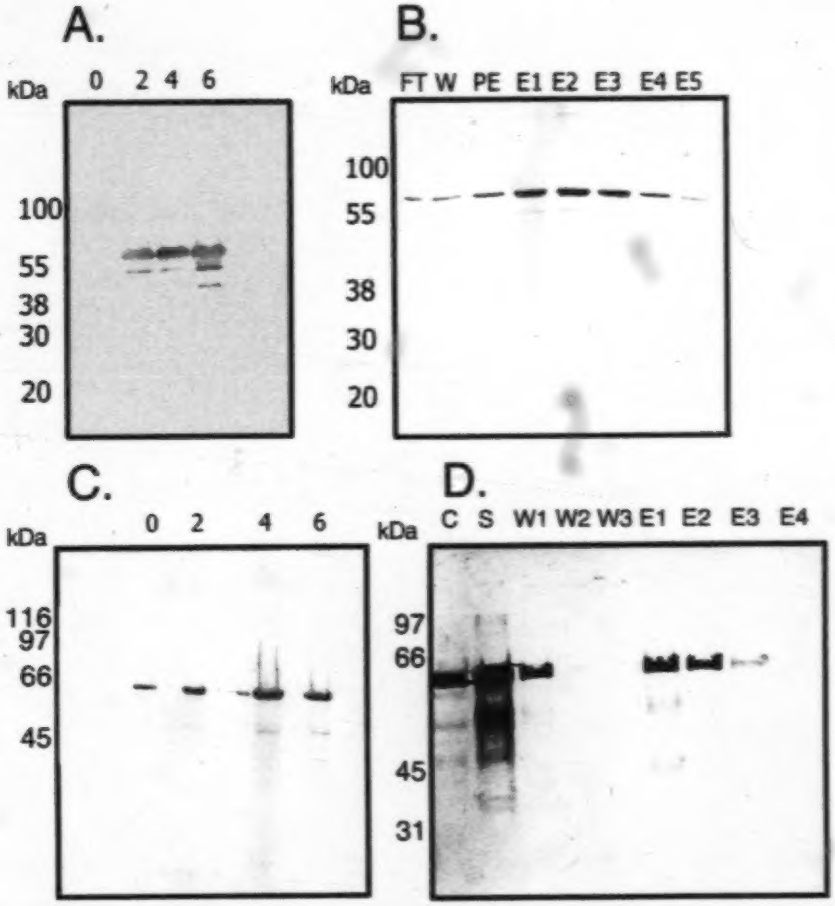
A.
kDa
0 2 4 6
100
55
38
30
20
B.
kDa
FT W PE E1 E2 E3 E4 E5
100
55
38
30
20
C.
kDa
0 2 4 6
116
97
66
45
D.
kDa
C S W1 W2 W3 E1 E2 E3 E4
97
66
45
31

Figure 5-7. Detection of the expression of recombinant α- (A and B)and β- (C and D) tubulin in *E. coli* cell lysates following pilot expression studies (A and C) and following purification using nickel-agarose affinity chromatography (B), or magnetic separation D). Pilot time course of expression of recombinant α- and β-tubulin by *E. coli* following induction with 1 mM IPTG at time 0 was analyzed using immunoblotting. A&B-sub-samples of bacterial cell cultures were withdrawn at 2, 4 and 6 hours following IPTG induction. C-Samples of the flow through (FT), wash (W), pre-elution (PE), and five elution (E1-5) fractions from the Ni-agarose column. D-samples of bacterial cells (C), supernatants of lysed bacteria (S), washes (W1-3), and elutions (E1-4) of magnetically separated nickel beads. All samples were analysed for the presence of recombinant proteins by SDS-PAGE and Western blotting using anti-HisG antibody.

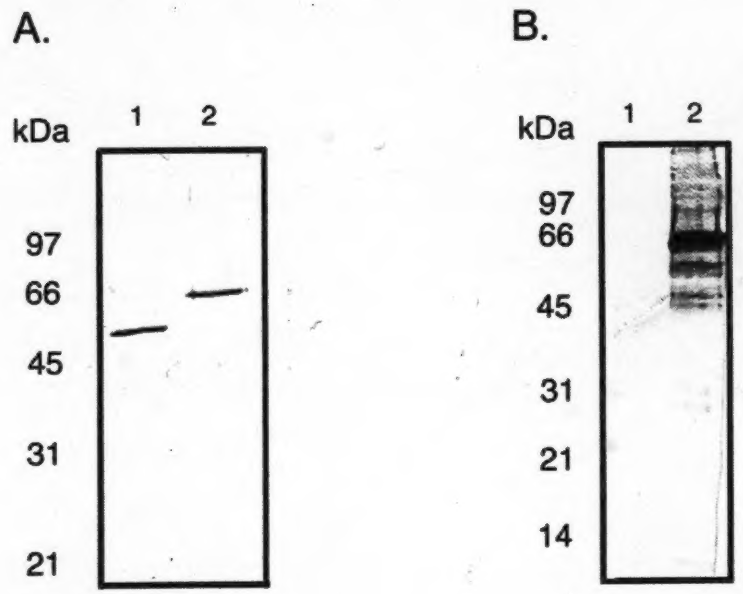
A.
B.
kDa
1
2
97
66
45
31
21
kDa
1
2
97
66
45
31
21
14

Figure 5-8. Reactivity of rabbit-anti-recombinant α-tubulin IgG with *T. danilewskyi* tubulin. *T. danilewskyi* tubulin in whole cell lysates (A1) as well as recombinant α(A2) and β (B2) tubulin, but not tubulin in goldfish cell lysates (B1) react with the αpolyclonal rabbit-anti- recombinant α-tubulin IgG (diluted 1:500).

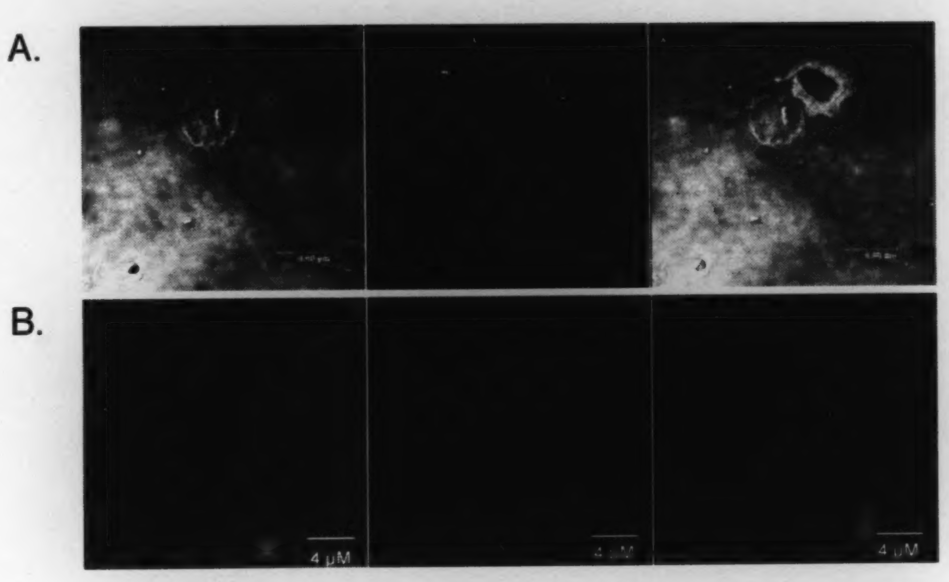

Figure 5-9. Immunofluorescence assay using anti-recombinant α-tubulin IgG and *Trypanosoma danilewskyi* isolated from goldfish blood. Trypanosomes were fixed to glass slides, permeabiilized, and incubated with rabbit anti-recombinant α-tubulin IgG (A) or isotype control (B) diluted 1:100 in PBS followed by incubation with FITC-conjugated goat anti-rabbit IgG diluted 1:100 in PBS. Differential Interference Contrast (DIC) (left panel), fluorescence (centre panel) and composite (right panel) images are shown for both. Slides were visualized using DIC imaging (40X objective lens) with a Leica confocal microscope.

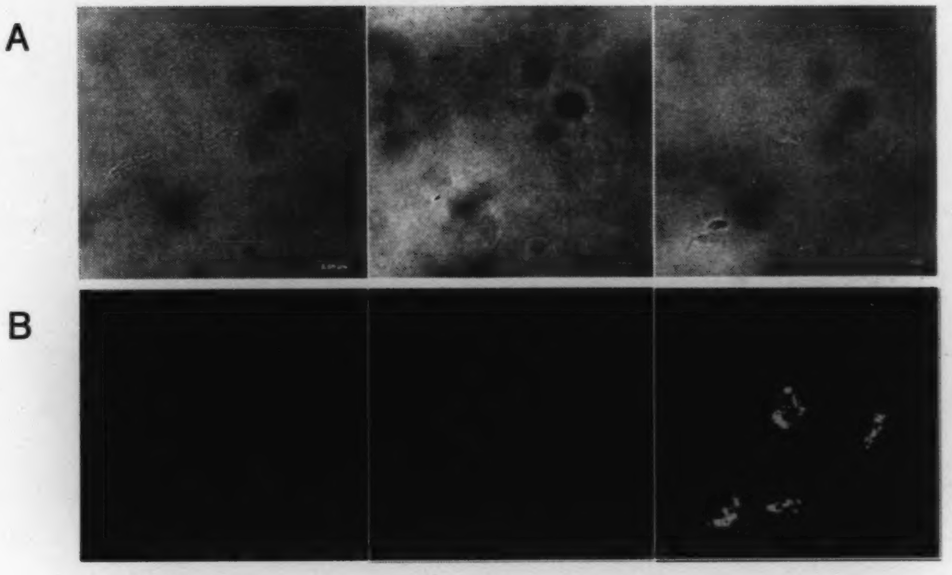

Figure 5-10. Immunofluorescence assay using anti-recombinant α-tubulin IgG and cultured *Trypanosoma danilewskyi.* Parasites were incubated with PBS (left), 80 μg IgG (center), or 160 μg IgG (right) for 2 days prior to fixation on glass slides. Following fixation the trypanosomes were incubated with FITC-conjugated goat anti-rabbit IgG diluted 1:100 in PBS. Differential Interference Contrast (DIC) (A), fluorescence (B). Slides were visualized using DIC imaging (40X objective lens) with a Leica confocal microscope.

CHAPTER 6

IMMUNIZATION OF GOLDFISH WITH RECOMBINANT *T. danilewskyi* α- and β-tubulin, AND THE EFFECTS OF RABBIT ANTI-TUBULIN AND GOLDFISH ANTIBODIES ON PARASITE GROWTH *IN VITRO*

6.1 Introduction

Several researchers have documented the long-term resistance of goldfish and carp to secondary infection with *T. danilewskyi* [389, 403]. This observation, in addition to the results of passive transfer studies involving serum or IgM from recovered hosts, and the capacity of fish to produce parasite-specific antibodies during infection, suggests that immunity to *T. danilewskyi* may be antibody-mediated [283, 403]. Inasmuch as specific antibody responses are generated toward parasite-derived molecules, an extensive array of trypanosome antigens have been evaluated in immunization trials for their ability to confer protection to naïve hosts [40]. Immunization of goldfish with excretory-secretory (ES) products of *T. danilewskyi*, particularly when administered in conjunction with Freund's complete adjuvant (FCA), resulted in significantly lower parasitemia following live parasite challenge in the first two weeks of the infection. Also, immunization with ES products led to a decrease in the prevalence of infection as compared to controls [43]. As a result of these studies, it was hypothesized that *T. danilewskyi* ES products contain molecules that stimulate a protective immune response in goldfish. Using Western blotting with serum pooled from infected fish, fractionated trypanosome ES products were shown to contain antigenic peptides that were homologous to *T. cruzi* α- and β-tubulin.

Tubulin from *T. brucei* has been shown to be immunogenic in mice. Immunization of mice has been performed with a *T. brucei* tubulin-rich fraction, denatured tubulin, synthetic tubulin peptides, and rat brain tubulin. The immunization with native tubulin (but not the denatured molecule, synthetic peptides, or rat tubulin) resulted in protection of the mice from challenge with *T. b. brucei*, *T. b. rhodesiense*, and *T. congolense*. Antibodies were implicated in the resistance of immunized mice to challenge infection because high levels of antibodies specific to native *T. brucei* tubulin, have been demonstrated [220]. This observation was further supported by the documentation of the passive transfer of immunity to naïve hosts by administration of serum from mice immunized with native *T. brucei* tubulin. In a related study, antibodies to each of the *T. brucei* tubulin preparations, the synthetic peptides, and rat tubulin were produced in rabbits. With the exception of the anti-rat tubulin antiserum, all of the anti-*T. brucei* tubulin sera inhibited the growth of parasites *in vitro*. The most effective antibodies were those that were generated towards native trypanosome tubulin, while those that were generated to the denatured tubulin and the synthetic tubulin peptides were less efficacious [221].

The purpose of the studies described in this chapter was to further evaluate the contribution of antibodies to the control of *T. danilewskyi* infection in the goldfish. Immunization of goldfish with recombinant trypanosome α- and β-tubulin was also performed to determine whether *T. danilewskyi* tubulin could induce a protective immune response in fish to live *T. danilewskyi* infection, similar to the response that was reported following immunization with ES products. Finally, the effects of affinity purified rabbit-anti-recombinant α-tubulin IgG and IgM from goldfish on the growth of *T. danilewskyi in vitro* were evaluated.

6.2 Experimental Design

6.2.1 Immunization of goldfish with recombinant *T. danilewskyi* α- and β-tubulin

T. danilewskyi α- and β-tubulin were identified as components of the parasite excretory-secretory (ES) products that stimulate antibody production in fish. ES products administered intraperitoneally (IP) in conjunction with Freund's complete adjuvant (FCA) were shown to be effective in protecting goldfish from challenge with *T. danilewskyi*, particularly within the first 2 weeks following infection. Similarly, immunization of mice with native *T. brucei* tubulin has been shown to protect against re-infection with a number of species of African trypanosomes. Therefore, *T. danilewskyi* α- and β-tubulin subunits were expressed as recombinant proteins in *E. coli* and used to immunize goldfish.

From previous immunization studies of goldfish with *T. danilewskyi* ES products, it was determined that the most effective route of immunization was IP in conjunction with an equal volume of FCA [40]. Therefore, immunization of goldfish with the recombinant tubulin subunits involved administration of 20 or 40 μg of the recombinant molecules IP in conjunction with FCA.

Since the recombinant proteins that were used for immunizations were expressed in *E. coli*, the samples containing the recombinant antigens were applied to a polymyxin B column to remove contaminating bacterial LPS (endotoxin). Samples were analyzed for the presence of endotoxin using a Limulus Amoebocyte Lysate (LAL) assay before and after application to the polymyxin B column. Using the LAL kit, it was determined that the samples used for immunization contained less than 0.25 endotoxin units (EU) of LPS/mL (endotoxin-free). Due to the anionic nature of the tubulin subunits, some of the recombinant protein was bound to the column and as a result portions of the samples were lost following this procedure. Therefore, some immunization trials involved IP administration of 20 and 40 μg of recombinant α- and β-tubulin + FCA in which the endotoxin was not removed. These samples were found to contain greater than 0.25 EU/mL using the LAL assay.

The fish used in the immunization experiments were 9.5-11cm in standard length. Blood samples were taken from each fish prior to immunization and examined for the presence of hemoflagellates. The number of trials for each immunization group, and the number of fish included in each trial, are listed in Table 6-1. Each trial consisted of the same number of fish in both the experimental recombinant α- and β-tubulin vaccination groups and the control groups. The control groups included: (1) fish that were not-immunized and not-infected to control for mortality not associated with treatment; (2) fish that were not-immunized but were infected with the same number of parasites to ensure infectivity of the challenge inoculum; and (3) sham-injected fish using a volume of sterile 1X PBS (vehicle for the recombinant proteins) and FCA equivalent to the volume administered to the vaccination groups. Like the experiments in which goldfish were immunized with trypanosome ES products, fish were challenged with 6.25×10^6 *in vitro* cultured *T. danilewskyi* 30 days after immunization. The effects of vaccination were determined by monitoring the number of parasites per millilitre of blood, volume of red blood cells, prevalence of infection, and mortality for each group on days 3, 7, 14, 21, and 28 post-infection. For experiments in which two trials were conducted, the data were pooled for analysis. Significant differences in parasitemia between experimental and control groups in each trial were determined using repeated measures one-way analysis of variance (ANOVA). For percent red blood cell volumes, a two-way ANOVA was performed since use of the repeated measures ANOVA would have excluded many individuals for whom some data points could not be obtained (*i.e.* leaked or broken capillary tubes). On each day, plasma samples were obtained from each fish and stored at -80°C. For trials in which there was a sufficient quantity of recombinant α- and β-tubulin remaining following vaccination of the goldfish, Western blots were performed using pre-immunization plasma samples, and plasma samples from 30 days post-immunization to determine whether immunized fish had generated antibodies specific to the immunizing antigen. Western blots were performed once for each immunization group outlined in Table 6-1 including the PBS control groups for each.

6.2.2 The effect of rabbit-anti-recombinant α-tubulin IgG on parasite growth *in vitro*

To determine whether anti-tubulin antibodies would affect the growth of *T. danilewskyi in vitro*, 1×10^5 parasites were incubated with various concentrations of affinity purified rabbit-anti-recombinant α-tubulin IgG for 7 days at 20°C. Each day, samples were withdrawn from the wells and the number of parasites remaining was enumerated once using a hemocytometer. Control treatments included wells containing complete culture medium only, and an isotype control consisting of affinity purified rabbit-anti-goldfish C3 IgG. The IgG for the isotype control was prepared in the same manner as the anti-recombinant α-tubulin IgG. Four independent experiments done in triplicate were performed for each treatment.

6.2.3 The effect of goldfish IgM from naïve and trypanosome infected goldfish on the growth of *T. danilewskyi in vitro*

In order to establish whether goldfish IgM had an effect on the growth of trypanosomes *in vitro*, a protocol was established to obtain partially purified and purified goldfish IgM from the serum of uninfected and *T. danilewskyi* infected fish. Immunoglobulin M was partially purified following precipitation of the serum with polyethylene glycol (PEG) 6000. The PEG-precipitated pellet contained goldfish IgM as determined by Western blotting and the precipitate was further fractionated using gel-permeation chromatography.

Fifteen large (>14cm), healthy goldfish were infected with 6.25×10^6 *T. danilewskyi* and 5 fish were exsanguinated on day 0, as well as on days 21 and 42 post-infection, as a source of serum IgM. The partially purified IgM was isolated from individual fish; however, equal quantities of protein from each fish were pooled prior to use in subsequent assays. For example, for the 20 μg treatment, 4 μg of partially purified IgM from each fish was combined prior to use in the assay. Due to the small volume of partially purified material recovered from the 5 infected fish (serum from days 21 and 42 post-infection), further purification of the PEG precipitated pellets containing IgM using FPLC was not possible. Since serum from uninfected (day 0) fish was more readily available, PEG precipitated IgM isolated from these fish was further purified by fractionation of the PEG-precipitated pellet based on size.

To determine the effects of the partially purified IgM from days 0, 21, and 42 post-infection on the growth of *T. danilewskyi in vitro*, 1×10^5 parasites were added to PBS containing a known concentration of IgM from each day post-infection. Control wells consisted of PBS only and PBS that had also been submitted to the PEG 6000 precipitation procedure. Parasites were enumerated each day for 4 days using a hemocytometer and each well was counted once. Due to the small quantities of material, there were two wells per treatment in each of two independent experiments.

Since the initial results indicated that parasite viability may be affected by partially purified IgM isolated from healthy fish (showing no sign of hemoflagellate infection), IgM from uninfected fish was purified to homogeneity and tested for activity. One hundred thousand parasites were added to PBS containing different concentrations of purified IgM. Control wells consisted of PBS only and PBS that had also been submitted to the PEG 6000 precipitation procedure. The viability of the parasites in each well was monitored over 4 days as described above. The experiment did not continue past 4 days since the viability of the parasites in the control wells could not be maintained, even after the addition of 0.1% glucose.

The IgM that was purified from day 0 (non-infected) goldfish serum was also pre-absorbed with trypanosomes and re-assayed for activity. After 4 days of incubation to pre-absorb the IgM, the plates containing the purified IgM and the parasites were centrifuged to pellet the trypanosomes. The supernatants were carefully removed and stored at 4°C as pre-absorbed samples. Subsequently, fresh *in vitro* cultured trypanosomes ($1\text{x}10^5$) were added to the pre-absorbed samples and the number of parasites remaining in each well was determined using a hemocytometer (one count per well) each day for 4 days.

6.3 Results

6.3.1 Immunization of goldfish with parasite α- and β-tubulin subunits increases their resistance to *T. danilewskyi* infection

In general, the density of parasites and the prevalence of infection were the best indicators of vaccine efficacy. The course of infection in the not immunized groups was similar to what has been documented previously, including the description of the course of infection found in Chapter 4. For all trials, parasite induced mortality was low, even in the not-immunized groups. No fish from the not-immunized/not infected groups died in any of the trials. In the groups in which mortality was observed, some death was most likely associated with high parasitemia; however, others were likely unrelated to parasitemia (*i.e.* jumping out of the tank). In addition, the mean percent red blood cell volume was not different between experimental and control groups for all trials. Over the course of the experiment the values for percent red cell volume decreased for all groups; however, this effect was likely a result of the sampling procedure involving repeated anesthesia and bleeding as discussed in Chapter 4. For the experiment in which fish were immunized with 20 μg of endotoxin-free β-tubulin, the group that was administered the recombinant molecule had lower percent red cell volumes from days 7-28; however, the parasitemia was not significantly different from the PBS-injected or the not-immunized/infected controls.

Western blotting was performed using pooled goldfish plasma samples to determine whether the immunized fish generate antibodies that recognize the antigen that they were administered prior to challenge with a live *T. danilewskyi* infection. The identity of the recombinant proteins used for immunizations (endotoxin-free and containing LPS) was confirmed by probing the nitrocellulose with anti-recombinant α-tubulin IgG (that recognizes both recombinant subunits), and anti-HisG antibodies. In some cases recognition of the tubulin subunits by antibodies in goldfish plasma appeared stronger than in others; however, equal volumes of plasma from each fish but not equal quantities of protein were pooled for immunoblotting preventing quantitative or semi-quantitative analysis. The small volumes of plasma harvested from each fish on each day (<50 μL), and the small dilution required for Western blotting, prevented protein quantification of the plasma samples prior to use in the assay. For example, it is tempting to speculate that the lack of tubulin-reactive antibodies in some groups of fish that were administered the samples containing LPS was due to the inhibition of lymphocyte reactivity by LPS; however, such a hypothesis requires confirmation by a more quantitative method of analysis, such as an ELISA.

6.3.1.1 Immunization with endotoxin-free recombinant tubulin subunits

Immunization of goldfish with endotoxin-free recombinant α-tubulin

Immunization of goldfish with 20 μg of recombinant *T. danilewskyi* α-tubulin, in conjunction with FCA, resulted in a reduction in the average number of circulating parasites that was prominent in the first two weeks of infection and significantly different (1.5 log lower) from the PBS-injected controls on day 7 post-infection. In addition, immunization with 20 μg of recombinant α-tubulin resulted in a decrease in the prevalence of infection when compared to controls. While all PBS-control fish had established infections 3 days post-inoculation, *T. danilewskyi* was not detected in all of the α-tubulin immunized fish until 21 days post-infection. One fish died in the PBS control group prior to infection and one fish in the α-tubulin group died between 14 and 21 days post-infection. The course of infection in the PBS control group was not different from the not-immunized, infected fish (Figure 6-1). Although immunization of goldfish with 40 μg of recombinant α-tubulin also resulted in a decrease in average number of parasites/mL of blood, particularly early in the infection the mean was not significantly different from that of the PBS-control group. A decrease in the prevalence of infection in fish immunized with 40 μg of recombinant α-tubulin was also observed until 21 days post-infection after which the number of undetectable infections in both groups was the same (Figure 6-2).

Immunization with endotoxin-free recombinant β-tubulin

Immunization of goldfish with 20 μg of endotoxin-free recombinant β-tubulin did not result in a decrease in the average parasitemia when compared to the group of fish injected with PBS (Figure 6-3). However, those fish receiving the recombinant molecule appeared to experience a peak in parasite levels later in the infection (day 21) than the control groups (day 7). Comparison of the prevalence of infection between the group of fish immunized with 20 μg of recombinant β-tubulin and the control groups revealed that it also took longer for all of the β-tubulin immunized fish to become infected. The mortality in the group that was administered 20 μg of β-tubulin was greater (20%) than the PBS control group in which no fish died. However, this fish died because it jumped out of the tank and was known to be harboring low number of parasites prior to its death. In addition, the cumulative mortality was not different from the not-immunized infected group (Figure 6-3).

Immunization of fish with 40 μg of endotoxin-free recombinant β-tubulin was more efficacious than the immunization with 20 μg of the same protein (Figure 6-4). Fish that were administered 40 μg of recombinant β-tubulin had lower levels of circulating parasites than PBS controls until 28 days post-infection. There was a greater than 2 log decrease in parasitemia on day 7 post-infection that was significantly lower than the PBS controls. The course of infection in PBS injected controls was not different from the course of infection in not immunized fish. In addition, the prevalence of infection in fish immunized with β-tubulin was also less than that of the controls over the course of infection (Figure 6-4).

Western blotting of the administered antigen preparations with plasma from goldfish immunized with endotoxin-free recombinant α- and β-tubulin

For the fish that received endotoxin-free recombinant α- and β-tubulin, as well as the PBS controls, antibodies to the recombinant tubulin subunits were detected in both the pooled pre-immunization and 30 day post-immunization plasma samples. All antibodies recognized a molecule of approximately 60 kDa, the size of the recombinant tubulin subunits with the appended vector sequence (Figure 6-5). Each individual blot is of the same sample of endotoxin-free recombinant α- or β-tubulin preparation separated on the same gel with the exception of the β-tubulin preparation reacted with the rabbit-anti-recombinant α-tubulin IgG, which was separated on a different gel.

6.3.1.2 Immunization with recombinant tubulin subunits containing LPS

Immunization of goldfish with recombinant α-tubulin containing LPS

In contrast to the results of the immunizations involving recombinant α-tubulin in which LPS had been reduced to less than 0.25 EU/mL using the polymyxin B column, immunization of fish with 20 or 40 μg of recombinant α-tubulin containing ≥0.25 EU/mL did not result in a decrease in the average number of parasites/mL of blood, or a decrease in the prevalence of infection (Figure 6-6). When fish were immunized with 20 μg of recombinant α-tubulin + LPS, there was a decrease in prevalence of infection on day 21 post-infection. However, the decrease in prevalence was not greater than the PBS control group on day 28 post-infection (Figure 6-6). There was no increase in mortality in the groups receiving recombinant α-tubulin + LPS compared to the groups that were administered the endotoxin-free samples.

Immunization with recombinant β-tubulin containing LPS

The results of the immunization studies in which goldfish were administered 20 μg of recombinant β-tubulin containing LPS are different from those in which fish were administered 20 μg of endotoxin-free β-tubulin, or α-tubulin containing LPS (Figure 6-7 A). Fish that were injected with 20 μg of β-tubulin containing LPS had significantly lower average levels of parasites than PBS controls on days 3, 7 and 14 post-infection. The difference between the experimental and PBS control group amounted to a 2 log decrease on days 7 and 14 after challenge. These denisities were not significantly lower than the not immunized fish and there was no difference in the course of infection between the PBS or β-tubulin-injected fish, and the not-immunized control group. Fish that were administered 20 μg β-tubulin containing LPS also had a lower prevalence of infection compared to the controls and one fish had not developed a detectable level of circulating parasites after 28 days of infection (Figure 6-7 A). This result is unusual because all fish that were not immunized but were infected in all of the immunization trials performed in this study were positive for trypanosomes at some point during the course of infection. It is uncertain whether the lack of infection was a result of an immunological phenomenon or a procedural error. Finally, there was no difference in the levels of parasites or prevalence of infection in fish immunized with 40 μg of β-tubulin containing LPS when compared to controls (Figure 6-7 B). As mentioned previously, one fish injected with 20 μg of endotoxin-free β-tubulin died (that was likely not related to trypanosome infection) while there was no mortality in either β-tubulin group that contained LPS.

Western blotting of the administered antigen preparations with plasma from goldfish immunized with recombinant α- and β-tubulin containing LPS

Plasma samples from fish immunized with the LPS-contaminated recombinant proteins were also tested for the presence of antibodies specific to the immunizing agents (Figure 6-8 A, B). Plasma from fish that were immunized with 20 μg of recombinant α-tubulin + LPS contained antibodies that recognize the immunizing antigen prior to and following immunization (Figure 6-8 A). On the other hand, for the 40 μg group, specific antibodies to recombinant α-tubulin + LPS were only detected in the plasma of fish 30 days following immunization. In this case, sera from the PBS controls did not possess antibodies that recognize the recombinant α-tubulin molecule (Figure 6-8 A).

When plasma samples from fish that were administered the recombinant β-tubulin containing LPS were tested for the presence of antibodies to the immunizing antigen, antibodies were detected in the pre-immunization samples from the 20 μg, 40 μg, and PBS groups, and in the pre-infection samples from the 40 μg group (Figure 6-8 B). There was no reaction of antibodies in the pre-infection plasma samples from the 20 μg and PBS immunized group with the recombinant β-tubulin containing endotoxin when the original blot is examined (Figure 6-8 B).

All nitrocellulose strips have LPS-containing α- or β-tubulin from the same antigen preparation. However, lane 1 and lanes 2-9 were separated on different gels prior to transfer and are therefore represented by their own sets of molecular weight standards.

6.3.2 Inhibition of parasite growth *in vitro* by anti-recombinant α-tubulin IgG

The growth of parasites that were incubated with 40 μg or 80 μg of purified rabbit-anti-recombinant α-tubulin IgG was inhibited significantly when compared to the growth of trypanosomes incubated with lower concentrations of IgG (0, 10, and 20 μg), or when compared to parasites incubated with 80μg of an isotype control (rabbit-anti-goldfish C3 IgG) (Figure 6-9). There was a slight decrease in the number of parasites treated with 20 μg of purified IgG that resulted in significantly fewer parasites than the controls (no antibody and the isotype control) on day 7. There was a significant decrease in the number of parasites treated with 80 μg of anti-recombinant α-tubulin IgG after one day of incubation and no live parasites could be detected in these wells following 2 days in culture. When parasites were exposed to 40 μg of anti-recombinant α-tubulin IgG there were significantly fewer parasites following 2 days of culture when compared to controls and no viable parasites were detected after 4 days of incubation (Figure 6-9).

6.3.3 Purification of goldfish IgM and inhibition of trypanosome growth *in vitro* by PEG precipitated and superose 6 purified goldfish IgM

Partially purified goldfish IgM was obtained by precipitation of the immunoglobulin fraction of goldfish serum using 9% PEG 6000. Goldfish IgM was identified in the 9% PEG pellet using a monoclonal antibody from mouse that recognizes carp IgM and cross reacts with the 80 kDa goldfish immunoglobulin heavy chain (see materials and methods in Chapter 3). A small quantity of IgM could also be detected in the 9% PEG 6000 supernatant (Figure 6-10 C). The pellet containing the partially purified goldfish IgM was further fractionated based on size using GP-FPLC. Purified goldfish IgM was detected in the second peak (pooled fractions 16-20) by silver staining, which revealed the presence of the heavy (80 kDa) and light (25 kDa) chains of IgM (Figure 6-10 D), and by Western blotting using the mouse-anti-carp IgM monoclonal antibody (Figure 6-10 E). Further analysis revealed the presence of the IgM in fractions 17-19 specifically (data not shown).

Partially purified (PEG-precipitated) IgM from day 0 as well as from days 21 and 42 post-infection affected the viability of *T. danilewskyi in vitro* (Figure 6-11). Surprisingly, the partially purified IgM from day 0 was the most effective in inhibiting parasite growth (Figure 6-11 A). There was a dose-dependent decrease in parasite number over 4 days. Incubation with 200 μg of IgM resulted in a significant decline in parasite survival after only 24 hours in culture as compared to the PBS and PBS/PEG controls. No trypanosomes remained in culture after 2 days of exposure to 80, 100, and 200 μg of PEG precipitated IgM from day 0. Incubation with 40 μg of PEG precipitated IgM from day 0 resulted in a steady decline in trypanosome number culminating in a complete elimination of viable parasites after 4 days (Figure 6-11 A)

Exposure to 200 μg of PEG precipitated IgM from fish that had been infected for 21 days also resulted in a steady, significant decline in parasite number over the 4 day incubation period as compared to controls (Figure 6-11 B). No viable parasites could be detected when samples were enumerated on day 4. Growth of *T. danilewskyi* exposed to 40, 80, and 100 μg of partially purified IgM from day 21 post-infection was not different from parasites exposed to control solutions (Figure 6-11 B).

When *T. danilewskyi* were incubated with 40, 80, 100, and 200 µg of PEG precipitated IgM from fish 42 days post-infection there was also a dose and time dependent decrease in parasite number (Figure 6-11 C). Exposure of trypanosomes to 200µg of partially purified IgM from day 42 resulted in a significant decrease in parasite number after 1 day in culture when compared to controls and no parasites could be detected after 2 days. A significant decrease in parasite number was detected in wells containing 100 µg of IgM from day 42 after 2 days of cultivation and almost no parasites remained on the 4th day. Likewise, a significant inhibition of trypanosome growth was observed in wells containing 80 µg of IgM when compared to controls after 3 days of cultivation, and no parasites could be detected on the 4th day of cultivation. Parasite growth in wells containing 40 µg of PEG precipitated IgM from 42 days post-infection was not different from that of the control wells over the 4 day observation period (Figure 6-11 C).

Since the partially purified IgM from day 0 was also effective in inhibiting parasite growth in vitro, IgM from healthy hosts was purified further and added to *T. danilewskyi in vitro*. There was a dose and time dependent decrease in the growth of the trypanosomes over 4 days of cultivation (Figure 6-12 A). The addition of 80, 100, or 200 µg of purified IgM from day 0 resulted in complete elimination of viable parasites after 1 day in culture. After 24 hours of cultivation there was also a significant decrease in parasite number in the wells containing 40 µg of purified IgM when compared to controls. No parasites remained in these wells after exposure to the IgM for 2 days. Parasite viability in the control wells declined to 0 after 3 days in culture and the decrease in the number of trypanosomes in wells containing 10µg of purified IgM was not different from the PBS and PBS/PEG controls. No agglutination of parasites was observed in any of the wells containing partially purified or purified IgM from uninfected fish, or in the wells containing partially purified IgM pooled from fish 21 and 42 days post infection. Purified IgM that was pre-absorbed with *T. danilewskyi* did not inhibit the growth of the parasites in the same manner as the purified IgM that was not pre-absorbed. The average number of parasites remaining in each well containing the pre-absorbed IgM was not significantly different from the PBS and PBS/PEG control wells on any of the days of cultivation (Figure 6-12 B).

6.4 Discussion

The main goals of the studies described in this chapter were: (1) to better characterize the nature of antibody-mediated immunity of goldfish to *T. danilewskyi* infection; and (2) to determine whether immunization of goldfish with recombinant *T. danilewskyi* α- and β-tubulin could confer protection to challenge with a live trypanosome infection.

It has been suggested that kinetoplastid tubulin represents a good vaccine candidate to prevent or control parasite infection because it is relatively invariant between different strains and species [220, 299]. Despite the relative conservation of the tubulin α- and β-subunits throughout evolution, trypanosome tubulin displays some differences in primary sequence and possesses different binding affinities for classical tubulin ligands when compared to their vertebrate counterparts [270]. Recently, it has been shown that immunization of mice with a purified native *T. brucei* tubulin-rich fraction protects mice from heterologous challenge with a number of species of African trypanosomes [220]. The advantage of using recombinant tubulin subunits for the immunization studies described in this chapter is that there is no chance of other trypanosome proteins contaminating the antigen preparation. The disadvantage is that the whole tubulin molecule is not being administered to the naïve goldfish hosts. For the previously published studies in which mice were immunized with a *T. brucei* tubulin-rich fraction, it was demonstrated that protection of naïve hosts could only be achieved through administration of the native molecule and not by injection of denatured tubulin or synthetic tubulin-peptides. However, in the same study it was determined that antibodies were generated towards all of the parasite tubulin derived immunizing antigens [220]. It would be interesting to determine whether fish vaccinated with a combination of recombinant *T. danilewskyi* α- and β-tubulin would show an increase in resistance to trypanosome infection when compared to fish immunized with either protein individually.

The results of the immunization studies described in this chapter suggest that administration of recombinant *T. danilewskyi* α- and β-tubulin subunits, in conjunction with FCA, can increase the resistance of goldfish to infection with live trypanosomes. The mean density of parasites and prevalence of infection were the best indicators of vaccine efficacy. Immunization of fish with 20 and 40 μg of endotoxin-free recombinant α-tubulin, or 40 μg of endotoxin-free β-tubulin resulted in the development of lower average levels of circulating parasites over the course of infection than PBS-injected controls. The effect was more pronounced in the first week following infection. This result indicates that immunization with these preparations results in the rapid mobilization of immune defenses that act to limit the number of parasites that establish in the naïve host after challenge with live parasites. In addition, the decrease in the prevalence of infection in these groups suggests that in some fish the immune response that is stimulated by immunization can delay or prevent the appearance of parasites in the bloodstream. Immunization of fish with 20 μg of endotoxin-free recombinant β-tubulin was not as effective as the other treatments in limiting parasitemia. However, there was a moderate decrease in the prevalence of infection when compared to controls. This result may be because the β-tubulin subunit is not as antigenic as the α-subunit and therefore a greater concentration (*i.e.* ≥40 μg) may be required for induction of a protective response in vaccinated hosts. In fact it has been determined that the greatest differences among the *T. danilewskyi* tubulin subunit sequences and the sequences of other flagellates (and some vertebrates) lie in the C-terminus of the α-tubulin subunit. Immunogenicity of the tubulin molecules from other organisms has been linked to the areas of greatest sequence divergence [79].

Since the recombinant *T. danilewskyi* tubulin subunits were produced in *E. coli*, the presence of contaminating endotoxin may have influenced the outcome of the vaccination studies. Bacterial LPS has different effects on the mammalian immune response that are concentration dependent. At low concentrations it can directly stimulate B-cells in the absence of T-cell help, facilitating the antibody response to T-independent antigens. In high concentrations, it can stimulate intense inflammatory reactions that may have perilous consequences for the host if they are not controlled. The immune responses that are associated with inflammation can antagonize or suppress lymphocyte activity and therefore may inhibit antibody production [200]. In fish, LPS is known to stimulate the activity of both B- and T-lymphocytes; however, the levels that are either immunostimulatory or toxic *in vivo* are unknown [183]. While the exact concentration of LPS in the preparations of purified recombinant α- and β-tubulin could not be determined exactly (due to the limited quantity of recombinant protein), the relative concentrations were determined using an LAL agglutination test with a sensitivity of 0.25 EU/mL.

The results of the immunization of goldfish in which LPS was not removed (>0.25 EU/mL) are not conclusive. In the case of immunization with 20 μg of α-tubulin and 40 μg of α- or β-tubulin, there was no difference in parasitemia, or in the prevalence of infection as compared to controls. This result is in contrast to the observation of increased resistance in fish administered the same quantity of endotoxin-free (<0.25 EU/mL) recombinant proteins discussed above. However, immunization of goldfish with 20 μg of recombinant β-tubulin resulted in a significant decrease in circulating parasite levels and prevalence of infection when compared to controls. It is possible that the concentration of LPS in the 20 μg sample stimulated B-cell responses to the recombinant β-tubulin, while the concentration in the 40μg sample, which was from the same antigen preparation, resulted in the suppression of specific antibody responses since twice the concentration of LPS would be present in the 40 μg dose. Since 20 μg of endotoxin-free β-tubulin was not of sufficient quantity to stimulate resistance to trypanosome infection (but 40 μg of endotoxin-free β-tubulin was), additional lymphocyte activation may be required to generate a protective response to a smaller quantity of antigen. Notably, the concentrations of LPS in the samples did not appear to be toxic to the fish since no mortality was observed in any of the groups receiving antigen preparations that contained LPS.

Western blotting with pooled, diluted plasma from fish pre-immunization and pre-infection (30 days post-immunization) revealed that antibodies that react with *T. danilewskyi* tubulin are present in non-immunized and non-infected fish. These results were unexpected but are not unprecedented since natural antibodies to tubulin and other host molecules have been detected in a number of species including mammals and fishes [133, 165, 188, 287, 340]. The source of these antibodies is unknown; however, antibodies that cross-react with *T. danilewskyi* tubulin epitopes may be generated during an infection with another pathogen, or towards the hosts' natural flora.

Natural antibodies to tubulin have been hypothesized to play a role in the regulation of host tubulin polymerization, cell proliferation, and the removal of tubulin from circulation following necrotic cell death [188]. Anti-tubulin antibodies have also been detected in the serum of patients infected with a number of different types of pathogens [165, 287]. The anti-tubulin antibodies that are generated during infection with *T. cruzi* are believed to contribute to the autoimmune-type pathology associated with Chagas' disease [185, 194]. It is not known if goldfish natural anti-tubulin antibodies also recognize goldfish tubulin, or if the natural antibodies bind to trypanosomes specifically. While the constitutive presence, or the infection-induced generation of antibodies that react with the hosts own proteins, is thought to be associated with autoimmunity, the possibility of cross-reactivity with trypanosome molecules, and therefore a possible role in the control of trypanosome infections, has received little attention in other systems.

Tubulin is immunogenic in a number of species and anti-tubulin antibodies that are induced by immunization have been shown to recognize different epitopes of the molecule than the natural antibodies [234, 364]. While the natural antibodies recognize internal epitopes that are not exposed under normal conditions, the antibodies induced by immunization recognize exposed sequences at the C-terminus of the molecule [188]. Since antibodies to *T. danilewskyi* tubulin were present in the plasma of the fish prior to immunization, it is not possible to determine whether immunization resulted in the induction of parasite-specific antibodies. However, it has been shown that immunization with exoantigens from *T. cruzi* augments a pool of polyreactive natural antibodies in mice [141].

Interestingly, goldfish have also been shown to possess natural antibodies to *Aeromonas salmonicida* A-protein, a major component of the bacterial cell membrane [339]. It was demonstrated that there is a large amount of variability in the concentration of natural anti-*A. salmonicida* A-protein antibodies within a population of goldfish, which affects individual susceptibility to infection-induced mortality [340]. Immunization of goldfish with an *A. salmonicida* surface protein has been shown to enhance the antibody response of individuals having low concentrations of natural antibodies, which effectively boosts resistance to infection [340]. Therefore, immunization of goldfish with *T. danilewskyi* ES products or recombinant tubulin subunits might enhance the concentration or affinity of the preexisting anti-tubulin antibodies. The requirement for FCA in the preparations of *T. danilewskyi* ES products, and recombinant tubulin subunits, for effective immunization suggests that T-cell responses play a role in the generation of a protective immune response. It is possible that the tubulin epitopes that are presented to T- and B-lymphocytes by APCs results in the generation of more specific, higher affinity antibodies to parasite tubulin than the pre-existing antibodies. In fact parasite tubulin has been shown to be a T-cell stimulating antigen during *Leishmania* infection [299].

To determine whether antibodies that are generated to *T. danilewskyi* tubulin would affect the growth of the parasites *in vitro*, known quantities of affinity-purified rabbit anti-α-tubulin IgG were added to trypanosome cultures. Concentrations of IgG greater than or equal to 40 μg were able to cause a dramatic decrease in the growth of the parasites over seven days of cultivation. The precipitous decline in parasite numbers that was observed for the 40 μg and 80 μg treatments was due to the presence of specific anti-tubulin antibodies since there was no decrease in parasite numbers observed in the control wells. These results are similar to what was observed when rabbit polyclonal antisera generated towards various *T. brucei* tubulin preparations were added to *T. brucei* in culture [221]. In addition to using purified IgG rather than whole rabbit serum, one difference between the experiments described in this chapter and those of Lubega *et al.* [221] was that no agglutination of trypanosomes was observed in wells containing α-tubulin-specific IgG. Antibody mediated agglutination was suggested to be the cause of parasite inhibition, based on their observation of complement-independent clumping of trypanosomes [221]. Although the precise mechanism of parasite death mediated by anti-recombinant α-tubulin IgG is unknown it has been observed that trypanosomes are capable of taking up antibodies into their cytoplasm [221]. We have also observed internalization of rabbit anti-recombinant α-tubulin IgG by *T. danilewskyi* (Chapter 5). Once the antibodies are taken into the cytoplasm, it is possible that they may inhibit formation of new microtubules and/or destabilize the microtubule structures involved in cell structure, motility, and division that are already present. The mechanism by which parasites take up antibodies is also not well understood; however, the existence of both antibody-specific and non-specific receptors/ligands present either on the surface or in the flagellar pocket of mammalian trypanosomes has been reported [61, 113, 382]. It is not known whether the natural anti-*T. danilewskyi* antibodies in goldfish serum also directly participate in the inhibition of growth of the parasites.

The results of the studies described above suggest that rabbit anti-*T. danilewskyi* recombinant α-tubulin antibodies can inhibit the growth of trypanosomes in culture through an unidentified mechanism that does not involve parasite agglutination.

The inhibitory activity of natural antibodies present in the serum of goldfish is supported by the results of the *in vitro* assays in which parasite growth was prevented in the presence of partially purified and purified IgM from uninfected fish. Partially purified IgM from fish infected for 21 and 42 days also inhibited parasite growth although the effect was only observed for the highest concentration of IgM from Day 21. The reason for this discrepancy is unknown; however, it may be speculated that during the acute phase of the infection, when the numbers of parasites in the blood are highest, polyclonal stimulation of B-cells occurs.

Polyclonal activation of lymphocytes is a characteristic of other mammalian trypanosome infections and has been implicated in the immunosuppression associated with *T. borreli* infection in carp [247, 317]. In fact it has been suggested that infection of carp with high numbers of *T. borreli* induces a higher level of unresponsiveness in isolated lymphocytes than infection with a smaller parasite concentration [317]. The hypothesis that *T. danilewskyi* might also stimulate polyclonal activation is supported by the observation of high antibody titres corresponding to peak levels of circulating parasites in goldfish and carp [43, 283]. Production of large quantities of irrelevant, non-specific antibodies would dilute the concentration of effective antibodies required for the elimination of the trypanosomes. Similar to the immune response of mammals to trypanosome infection, parasite-specific antibodies would eventually be produced, the non-specific antibodies would be bound and eliminated, and parasite numbers would be reduced to a manageable level.

Passive transfer of serum and IgM from fish that have recovered from *T. danilewskyi* infection has been shown to confer protection to naïve hosts [283, 403]. It would be interesting to determine whether the same effect could be achieved using serum isolated from fish during the acute phase of the infection. Alternatively, the effective antibodies could be bound by the high concentration of parasites present in the blood during the acute phase of the infection. In either event, the result would require larger quantities of purified IgM to achieve the same relative concentration of inhibitory antibodies.

The effect of the highest concentrations of purified IgM from uninfected fish was rapid with complete elimination of the parasites occurring within a day of incubation. Pre-absorption of the IgM with trypanosomes completely abrogates this effect. The mechanism by which parasite death occurs is unknown; however, no parasite agglutination was observed. These results are in contrast to the unpublished results of Overath *et al.* [283], who reported the observation of agglutination of fish trypanosomes incubated with purified carp IgM. The toxicity of the purified IgM is likely not related to complement activity since it has been shown that *in vitro* cultivated *T. danilewskyi* are not susceptible to complement-mediated lysis by goldfish serum, regardless of the presence of parasite-specific antibodies [296]. The possibility of another molecule co-purifying with the goldfish IgM has not been ruled out by the studies described in this chapter. Compounds other than the purified IgM that could inhibit parasite growth *in vitro* include lipoproteins, small molecules such as antimicrobial peptides that may have run off the gel or that have complexed with IgM, and molecules that are the same size as the heavy or light chains of goldfish IgM. Apolipoprotein L-1 was recently shown to be the lytic component of the trypanosome lytic factor (TLF) isolated from the sera of certain species of primates. TLF mediates immunity of humans and some other species of primates to infection with *T. b. brucei* when it is taken up by the parasites and causes parasite lysis [297, 378]. The results of the pre-absorption studies described above suggest that the molecule, whether it is IgM or another serum component, is bound or taken up by the parasites to exert its toxic effects.

The interpretation of the results of these studies was influenced by the variability introduced by limited sample sizes, and the differences in the immune responses of individual hosts from an out-bred population of fish to immunization and infection. In addition, variability was also introduced by the limits imposed as a result of the labor intensive and time-consuming enumeration procedures. The number of parasites from each sample, whether from a fish or from the well of a culture plate, could only be counted once since enumeration of large numbers of samples were required for most assays. Performing more than one hemocytometer count from each sample would undoubtedly improve the accuracy. Nevertheless, a number of conclusions can be drawn regarding the effects of rabbit and goldfish antibodies on parasite growth, and the effects of immunization of goldfish with recombinant *T. danilewskyi* α- and β-tubulin.

In this chapter, the effectiveness of vaccination of goldfish with recombinant *T. danilewskyi* α- and β-tubulin for increasing the resistance of naïve hosts to infection with a high concentration of parasites has been demonstrated. It has also been shown that goldfish possess antibodies that cross react with *T. danilewskyi* tubulin. Immunization of goldfish with the recombinant tubulin subunits may augment a pool of tubulin-specific B-cells that are already present in the fish. While the precise mechanism(s) remain to be elucidated, natural IgM purified from the serum of non-infected fish, and affinity purified rabbit-anti-α-tubulin IgG both prevent the growth of parasites *in vitro* in a manner that does not involve agglutination or complement mediated lysis of the parasites. Although partially purified IgM isolated from fish in the acute and recovery phases of *T. danilewskyi* infection also prevented the growth of parasites *in vitro* the effect was observed only for the highest concentration of IgM isolated from fish during the acute phase of the infection. The reason may be related to immunosuppression induced by the parasites, or absorption of the effective pool of antibodies by high concentrations of parasites.

These experiments represent the first evidence for the immunogenicity of trypanosome tubulin in fish and demonstrate the direct activity of fish antibodies on the growth of *T. danilewskyi in vitro*. Results suggest that the presence of natural and induced antibodies may be a mechanism by which fish are able to control parasitemia, particularly in the early stages of primary and secondary *T. danilewskyi* infections.

Table 6-1. Summary of the number of immunization trials involving the intraperitoneal administration of two concentrations of recombinant *Trypanosoma danilewskyi* α- and β-tubulin and the number of fish (n) used in each trial.

		20 μg	***n*[b]**	**40 μg**	***n***
Recombinant α-tubulin	**-LPS[a]**	2 trials	10	1 trial	5
Recombinant β-tubulin	**-LPS**	1 trial	5	1 trial	5
Recombinant α-tubulin	**+LPS**	1 trial	5	1 trial	5
Recombinant β-tubulin	**+LPS**	2 trials	13	2 trials	13

[a] In some experiments bacterial lipopolysaccharide (LPS) was removed (<0.25 EU/mL) using a polymyxin B column (-LPS), while in other experiments the LPS was not removed (+LPS).

[b] *n*=the number of fish used in each experimental and control group within the trial.

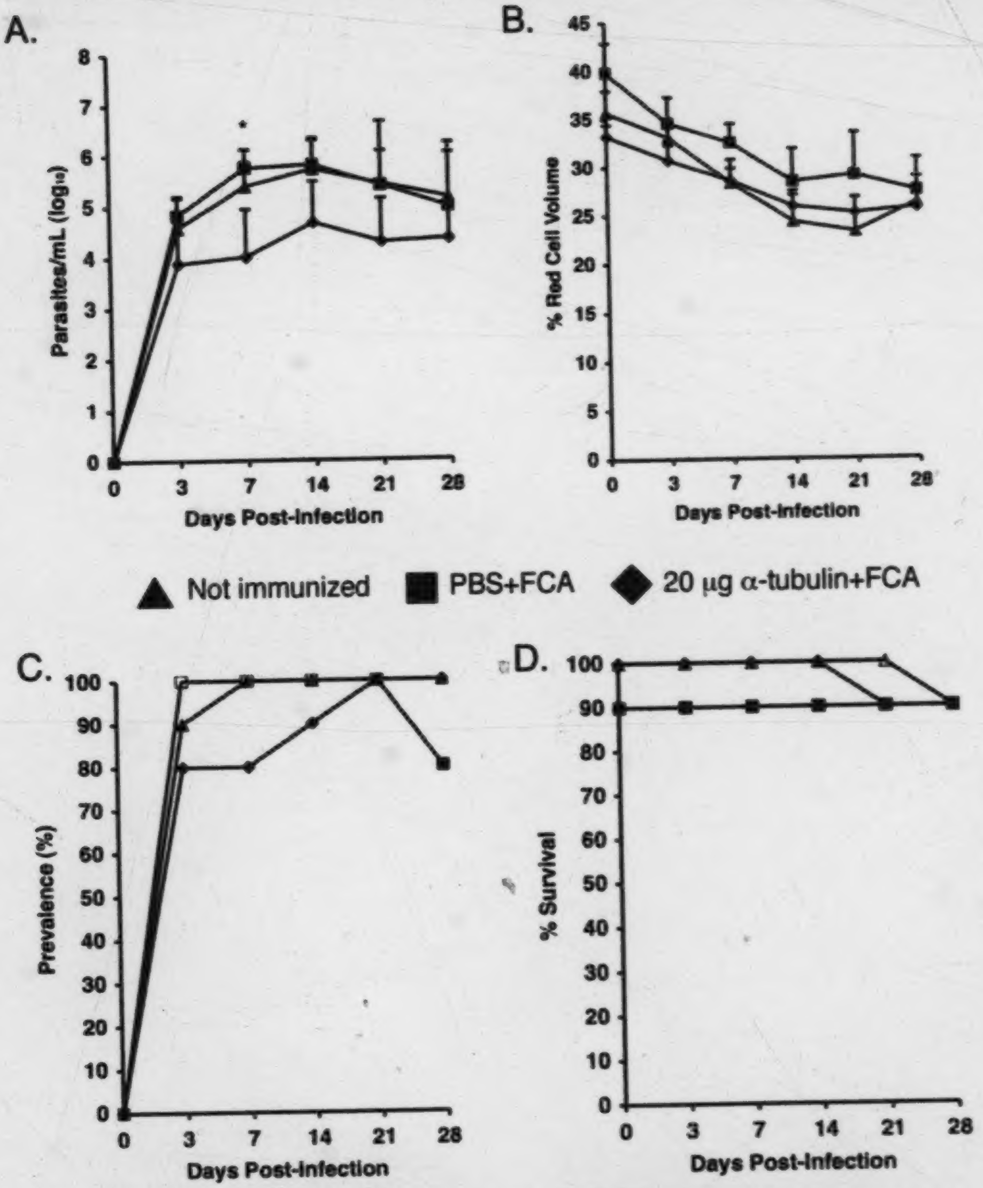

Figure 6-1. The effect of intraperitoneal administration of 20 μg of detoxified recombinant α-tubulin + Freund's complete adjuvant (diamond) or control solution (square) to goldfish 30 days prior to infection with $6.25\text{x}10^6$ *T. danilewskyi*. Values for non-immunized controls are provided for comparison (triangle). A-parasitemia; B-packed red blood cell volume; C-prevalence of infection; D-mortality. In A and B, points represent the mean± SEM of 10 fish from two trials.

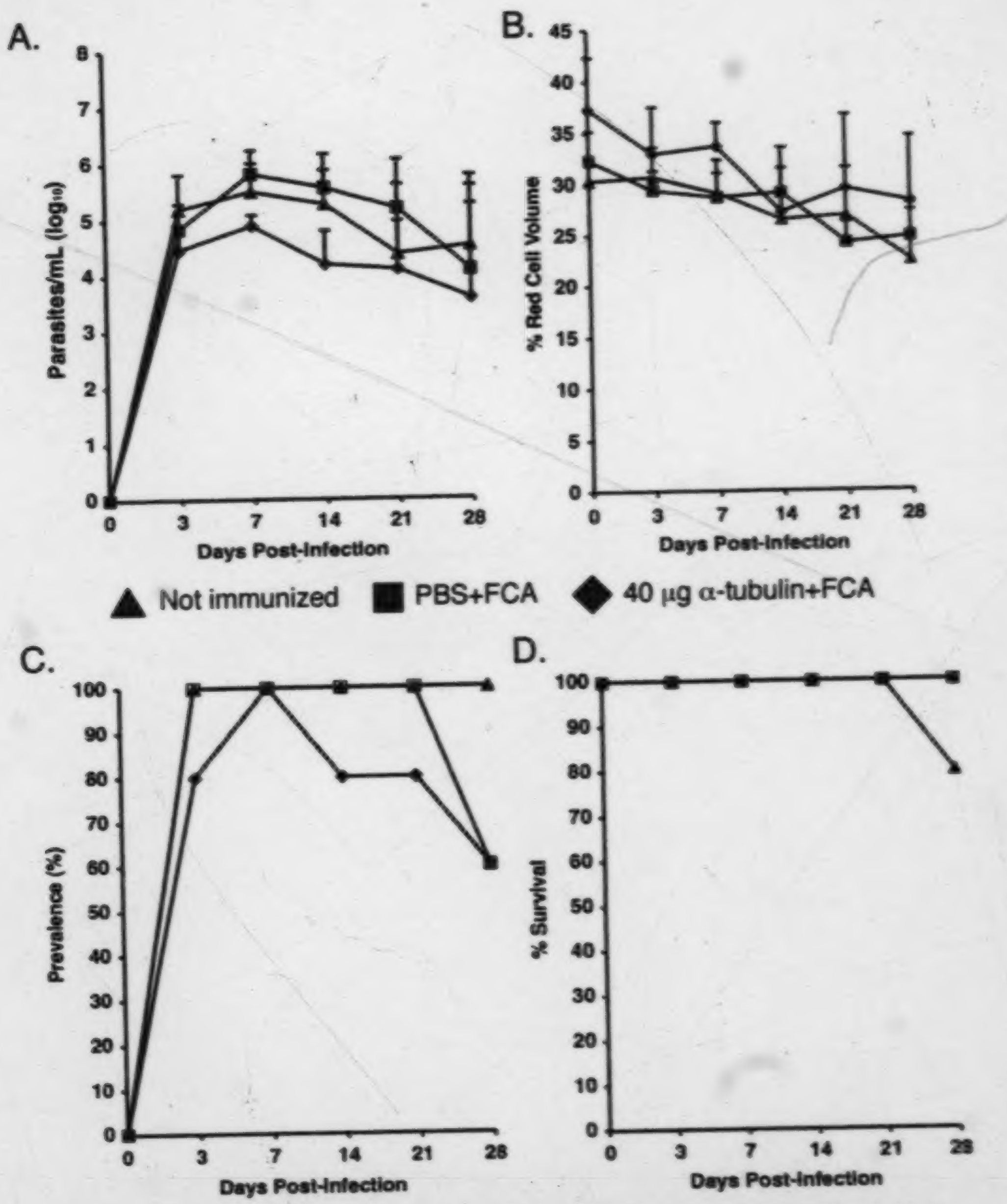

Figure 6-2. The effect of intraperitoneal administration of 40 µg of endotoxin-free recombinant α-tubulin+ Freund's complete adjuvant (diamond) or control solution (square) to goldfish 30 days prior to infection with 6.25×10^6 *T. danilewskyi*. Values for non-immunized controls are provided for comparison (triangle). A-parasitemia; B-packed red blood cell volume; C-prevalence of infection; D-mortality. In A and B, points represent the mean± SEM of 5 fish from one trial.

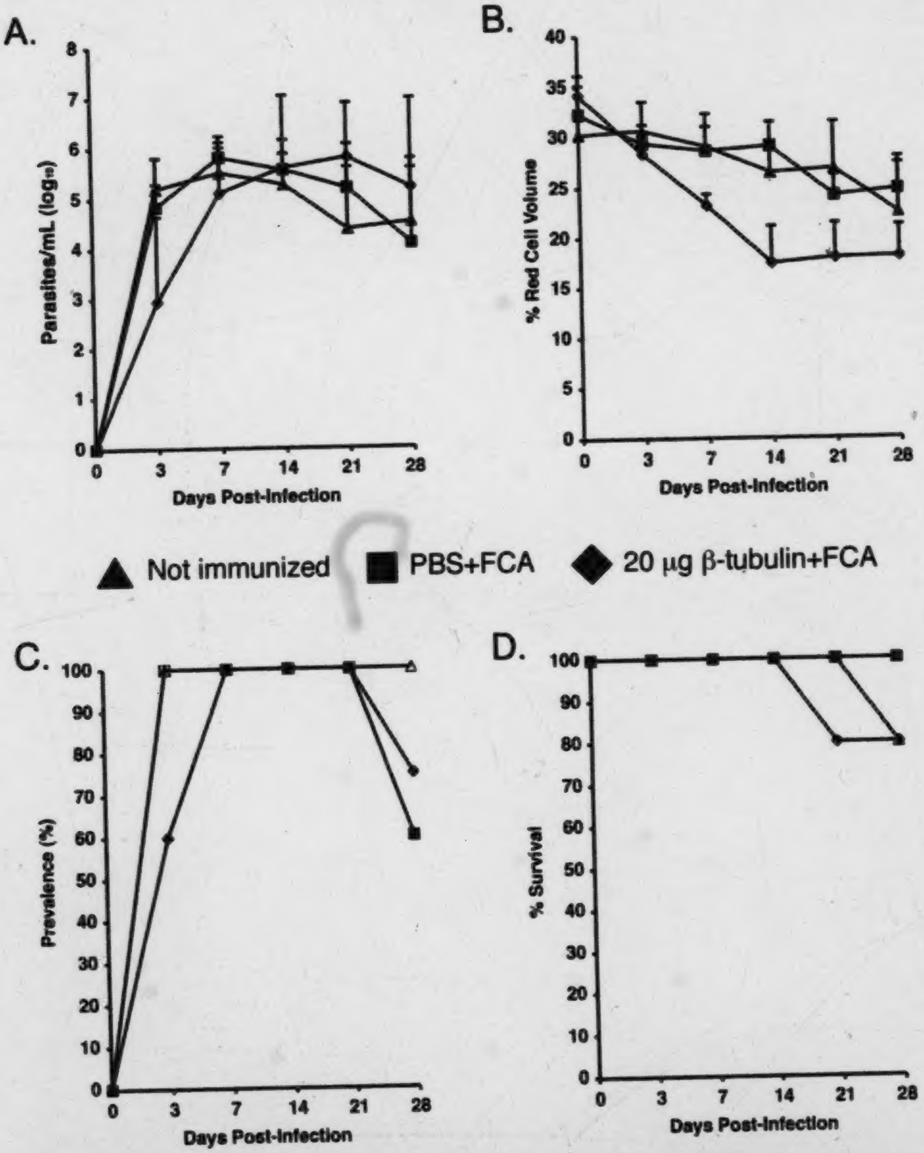

Figure 6-3. The effect of intraperitoneal administration of 20 μg of endotoxin-free recombinant β-tubulin + Freund's complete adjuvant (diamond) or control solution (square) to goldfish 30 days prior to infection with 6.25×10^6 *T. danilewskyi*. Values for non-immunized controls are provided for comparison (triangle). A-parasitemia; B-packed red blood cell volume; C-prevalence of infection; D-mortality. In A and B, points represent the mean± SEM of 5 fish from one trial.

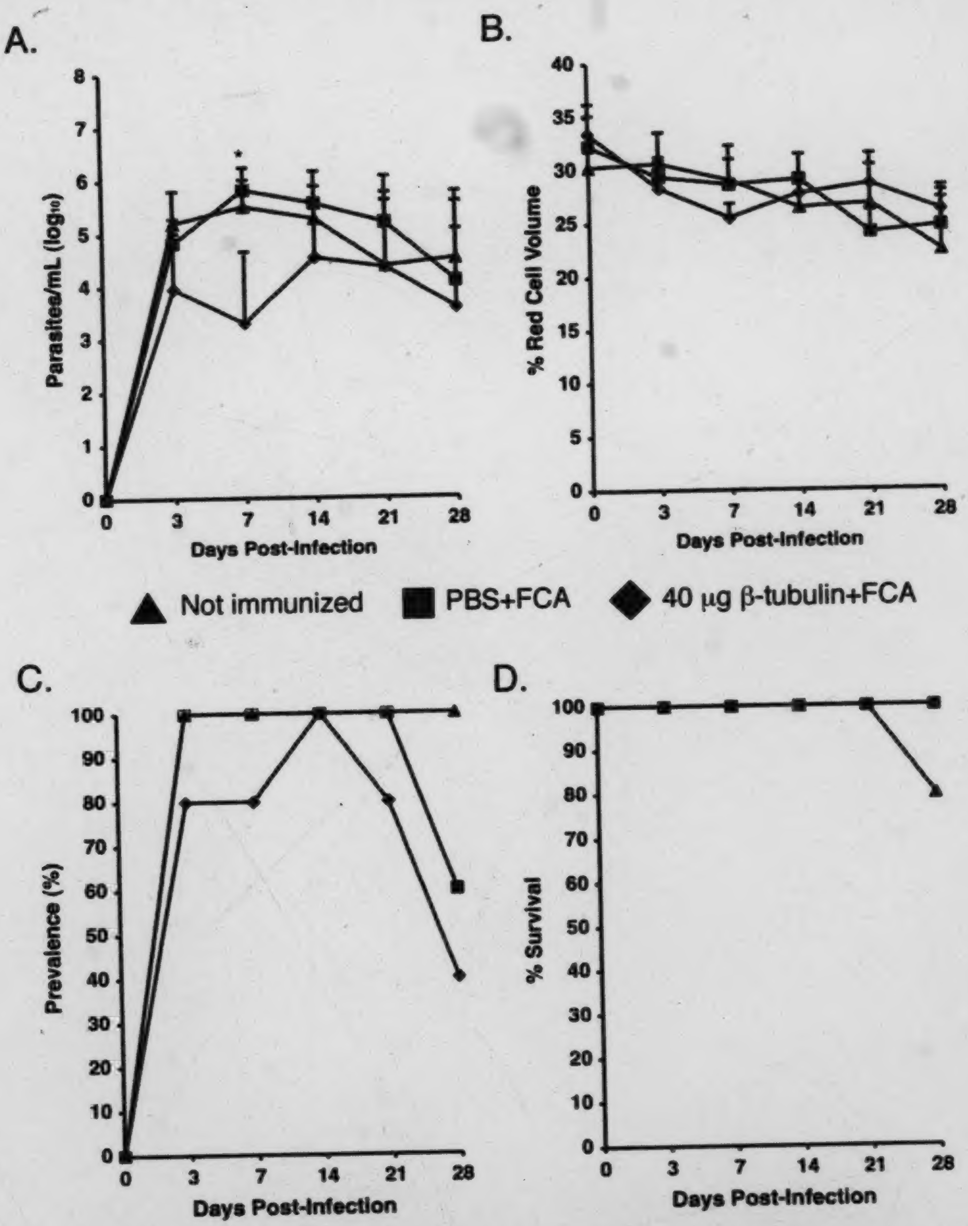

Figure 6-4. The effect of intraperitoneal administration of 40 µg of endotoxin-free recombinant β-tubulin + Freund's complete adjuvant (diamond) or control solution (square) to goldfish 30 days prior to infection with $6.25\text{x}10^{6}$ *T. danilewskyi*. Values for non-immunized controls are provided for comparison (triangle). A-parasitemia; B-packed red blood cell volume; C-prevalence of infection; D-mortality. In A and B, points represent the mean± SEM of 5 fish from one trial. * represents significant difference from PBS-immunized controls, P≤0.05

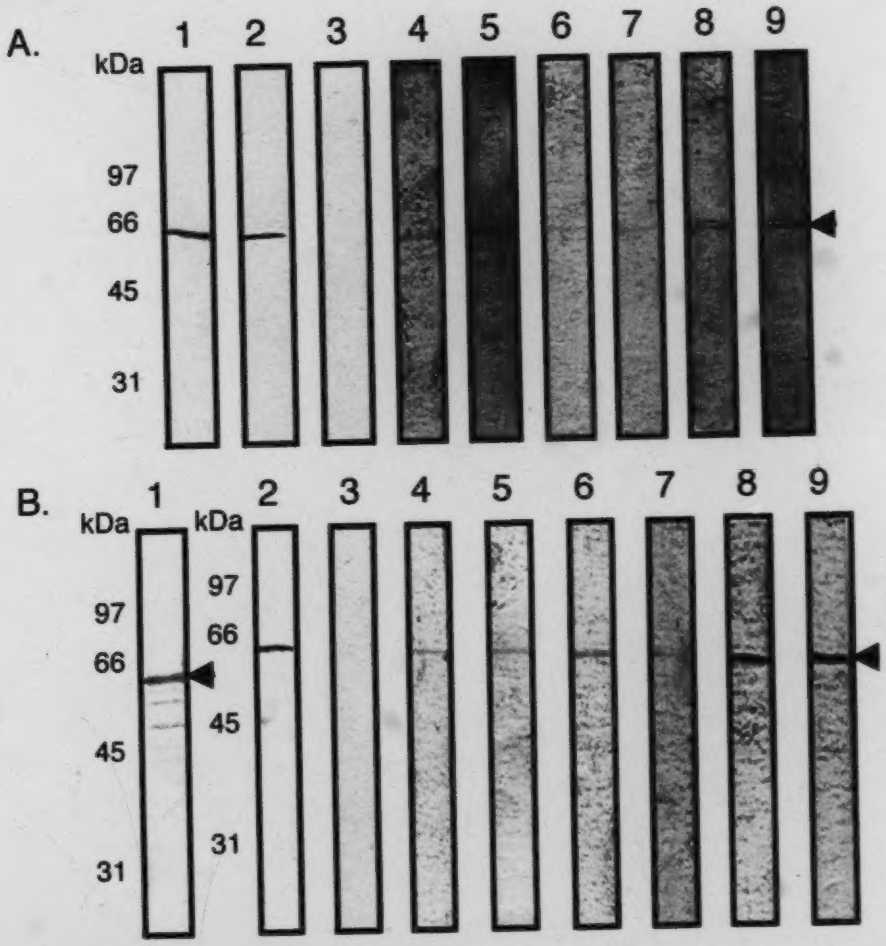

Figure 6-5. Goldfish immunized with endotoxin-free recombinant *T. danilewskyi* α-(A) and β-tubulin (B) have antibodies that recognize the immunizing antigen. The identity of the recombinant molecules used for immunization was confirmed using immunoblotting with rabbit anti-recombinant α-tubulin IgG (Lane 1) (diluted 1:400 in blocking buffer), and with anti-HisG antibody (Lane 2) (diluted 1:5000 in blocking buffer). Fish were immunized intraperitoneally with 20 and 40 μg of recombinant α-tubulin, β-tubulin, or PBS with an equal volume of Freund's complete adjuvant. An equal volume of serum from each fish belonging to each experimental group was pooled from samples collected prior to immunization (preI) and 30 days post-immunization (30 dpim). The presence of antibodies reacting with the parasite α-tubulin was determined using Western blotting (sera were diluted 1:25 in blocking buffer). Lane 3: no goldfish serum, Lane 4: PBS control preI, Lane 5: PBS control 30 dpim, Lane 6: 20 μg immunization preI, Lane 7: 20 μg immunization 30 dpim, Lane 8: 40 μg immunization preI, Lane 9: 40 μg immunization 30 dpim.

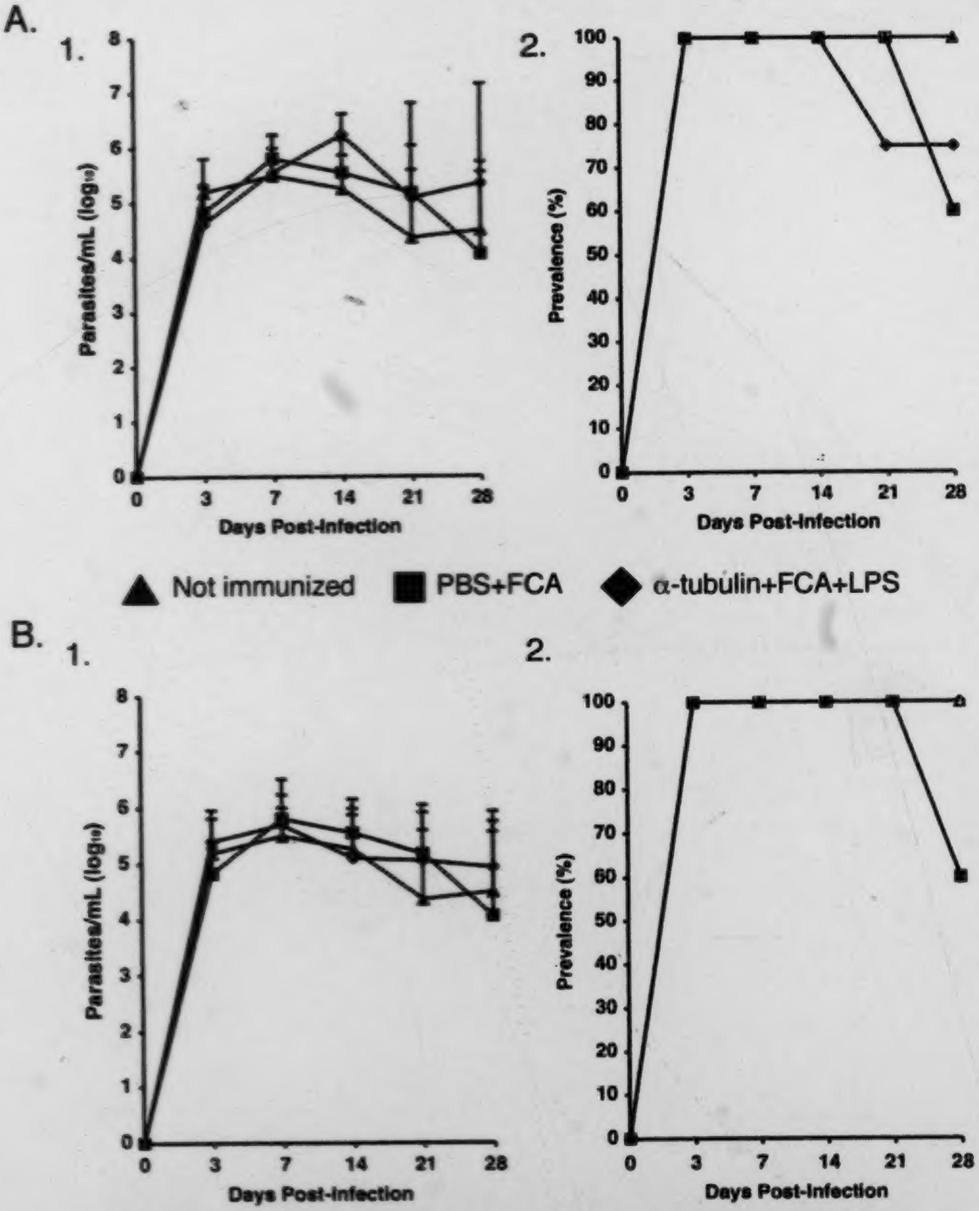

Figure 6-6. The effect of intraperitoneal administration of 20 μg (A) and 40 μg (B) of recombinant α-tubulin containing LPS + Freund's complete adjuvant (diamond) or control solution (square) to goldfish 30 days prior to infection with 6.25×10^6 *T. danilewskyi*. Values for non-immunized controls are provided for comparison (triangle). 1-parasitemia; 2-prevalence of infection. In A and B, points represent the mean ± SEM of 5 fish from one trial.

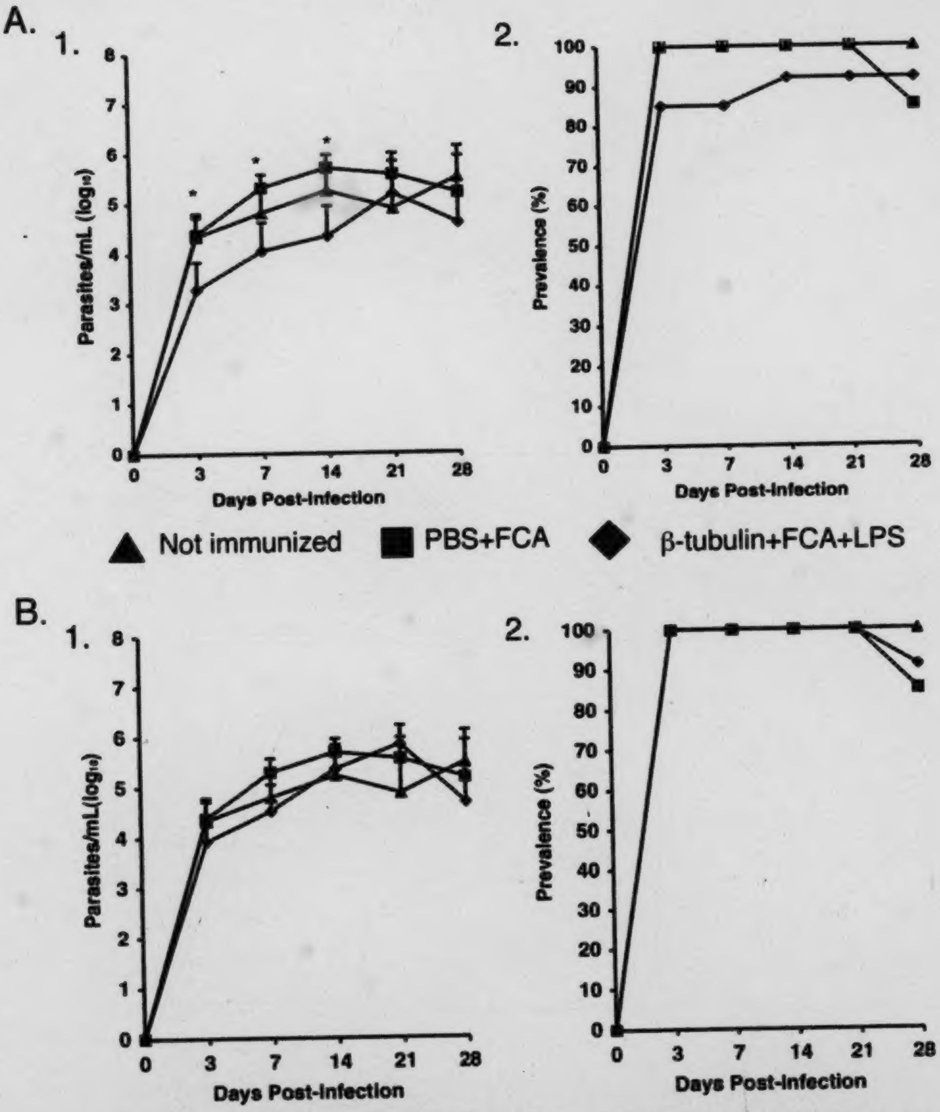

Figure 6-7. The effect of intraperitoneal administration of 20 μg (A) and 40 μg (B) of recombinant β-tubulin containing LPS + Freund's complete adjuvant (diamond) or control solution (square) to goldfish 30 days prior to infection with 6.25×10^6 *T. danilewskyi*. Values for non-immunized controls are provided for comparison (triangle). 1-parasitemia; 2-prevalence of infection. In A and B, points represent the mean ± SEM of 13 fish from two trials. * represents a significant difference from PBS-immunized controls, $P \leq 0.05$.

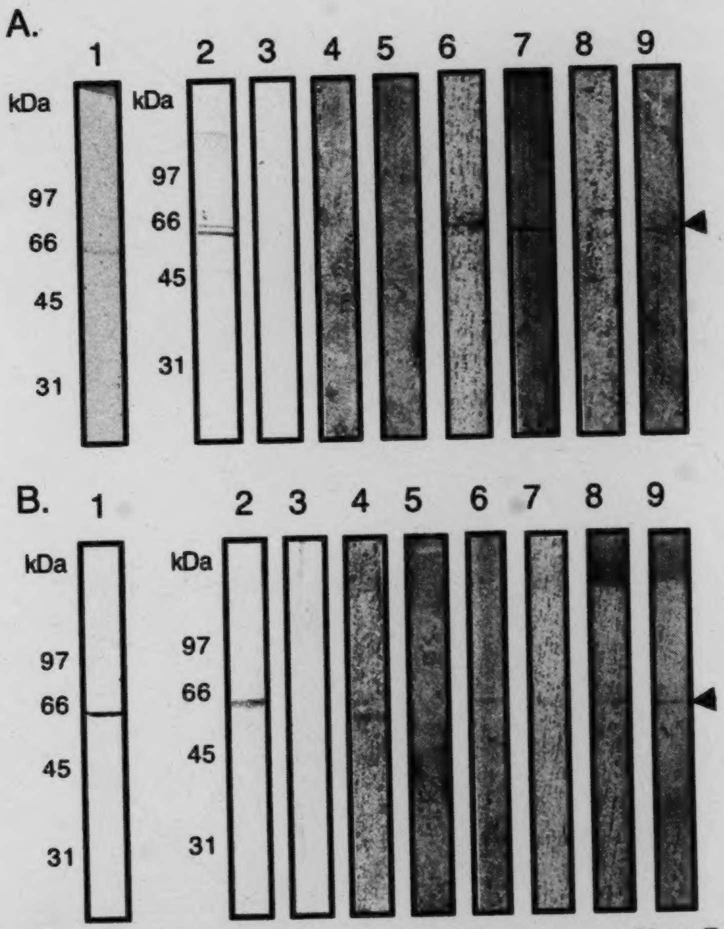

Figure 6-8. Goldfish immunized with endotoxin-containing recombinant *T. danilewskyi* α-(A) and β-tubulin (B) have antibodies that recognize the immunizing antigen. The identity of the recombinant molecules used for immunization was confirmed using immunoblotting with anti-HisG antibody (Lane 1) (diluted 1:5000 in blocking buffer) and, with rabbit anti-recombinant α-tubulin IgG (Lane 2) (diluted 1:400 in blocking buffer). Fish were immunized intraperitoneally with 20 and 40 μg of recombinant α-tubulin, β-tubulin or PBS with an equal volume of Freund's complete adjuvant. An equal volume of plasma from each fish belonging to each experimental group was pooled from samples collected prior to immunization (preI) and 30 days post-immunization (30 dpim). The presence of antibodies reacting with the recombinant parasite tubulin was determined using Western blotting (plasma was diluted 1:25 in blocking buffer). Lane 3: no goldfish plasma, Lane 4: PBS control preI, Lane 5: PBS control 30dpim, Lane 6: 20 μg immunization preI, Lane 7: 20 μg immunization 30 dpim, Lane 8: 40 μg immunization preI, Lane 9: 40 μg immunization 30 dpim.

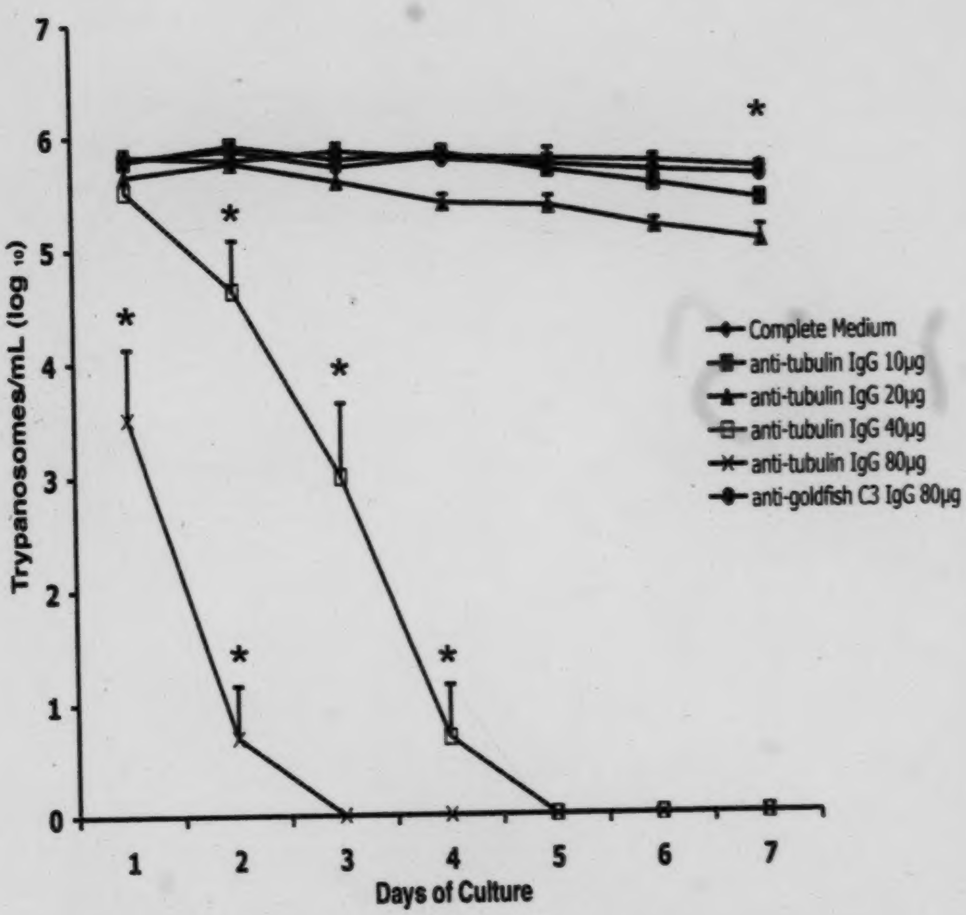

Figure 6-9. The effects of affinity-purified anti-α-tubulin IgG on the growth of *Trypanosoma danilewskyi in vitro*. Known concentrations of the anti-recombinant α-tubulin IgG were added to parasite cultures. Isotype control was affinity-purified anti-goldfish complement component 3 IgG. Plates were incubated at 20°C for 7 days and the number of viable parasites was determined using a hemocytometer. Data represent the mean of triplicate wells for four independent experiments ±SEM. * represents significant difference from complete medium control, P≤0.05.

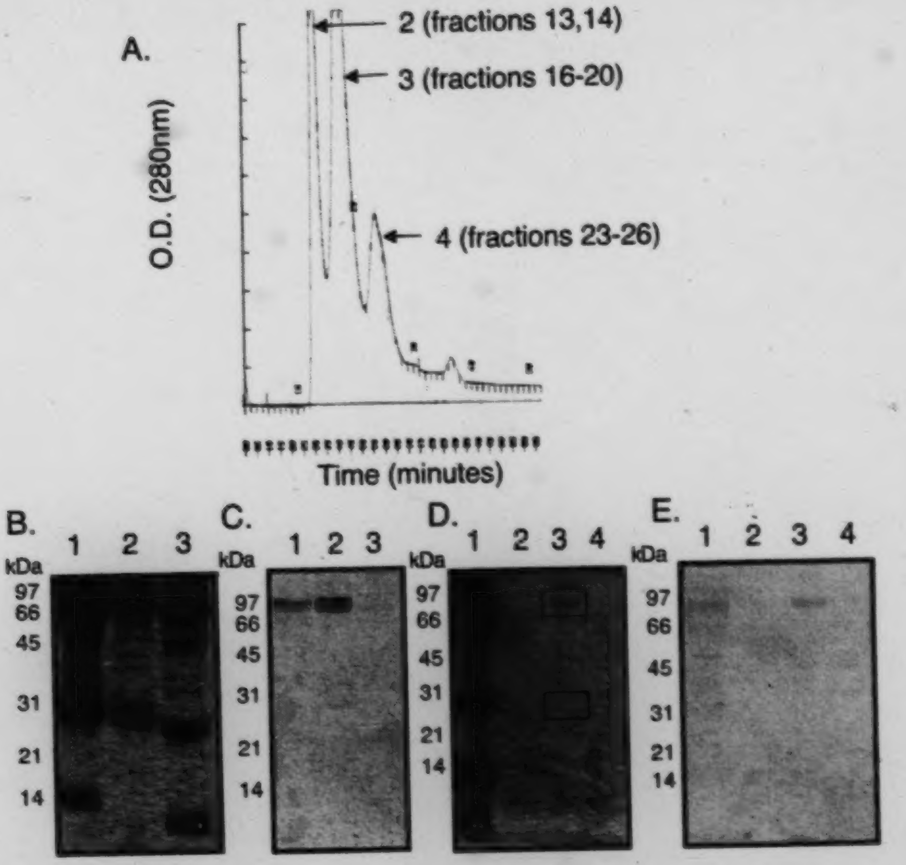

Figure 6-10. Purification of goldfish IgM using polyethylene glycol 6000 precipitation (B and C) and gel permeation chromatography (A, D, E). Serum was pooled from goldfish and the immunoglobulin fraction was isolated using 9% PEG. Unprecipitated serum (B1, C1), 9% PEG pellet (B2, C2), and the 9% PEG supernatant (B3, C3) were analyzed for the presence of goldfish IgM using SDS-PAGE followed by Coomassie blue staining (B) and Western blotting (C). IgM was detected in C and E using a monoclonal antibody to an epitope of the heavy chain of carp IgM (80 kDa). The PEG 6000 precipitated immunoglobulin fraction was applied to a superose 6 column and fractions were eluted in 1X PBS at a flow rate of 0.4 mL/minute. Fractions were pooled from the first 3 peaks (A) and analyzed for the presence and purity of IgM using SDS-PAGE followed by silver staining (D) and Western blotting (E). Lane D1, E1-unfractionated goldfish serum,; lane D2, E2-fractions 13,14; lane D3, E3-fractions 16-20; lane D4, E4-fractions 23-26.

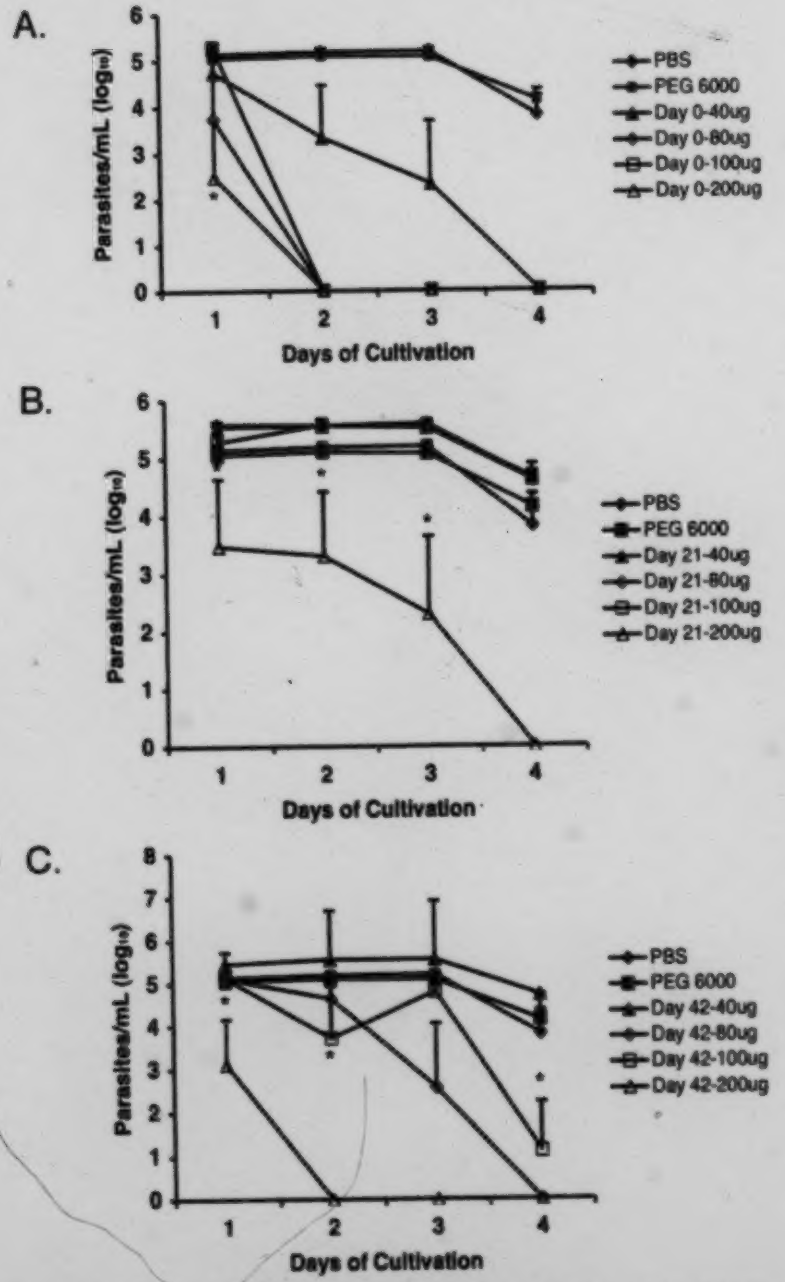

Figure 6-11. The effects of partially purified goldfish IgM on the growth of *T. danilewskyi in vitro*. 1×10^5 trypanosomes were added to various concentrations of partially purified IgM from uninfected fish (A) as well as partially purified IgM obtained from the pooled serum of goldfish 21 and 42 days post-infection (B and C). Control wells consisted of PBS only and PBS that had been submitted to the same PEG precipitation procedure as the different goldfish sera. Points represent the mean of two wells from two independent experiments±SEM. * indicates significant difference $P \leq 0.05$ when compared to controls.

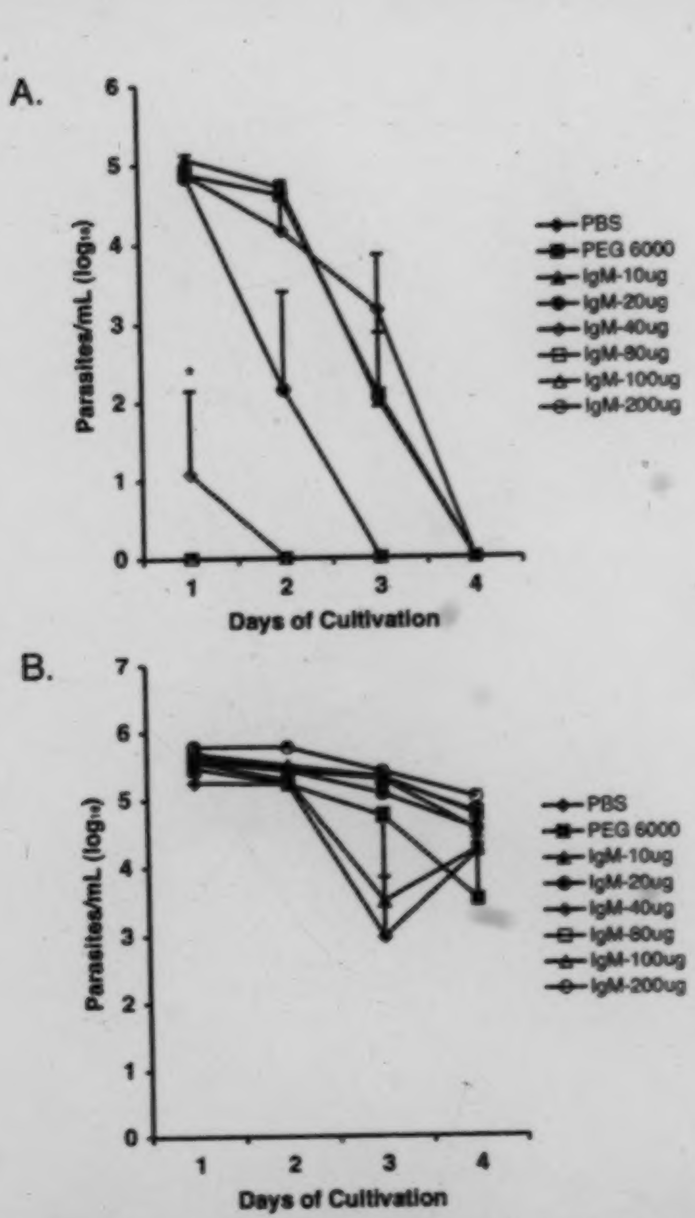

Figure 6-12. The effects of purified goldfish IgM (A) and pre-absorbed purified goldfish IgM (B) on the growth of *T. danilewskyi in vitro*. 1x10^5 trypanosomes were added to different concentrations of purified IgM from uninfected (day 0) fish. Control wells consisted of PBS only and PBS that had been submitted to the PEG precipitation procedure for partial purification of goldfish IgM. The number of parasites remaining in each well on each day was determined using a hemocytometer. Following 4 days of incubation with the parasites the supernatants were removed and mixed with freshly isolated *in vitro* cultivated *T. danilewskyi* . The survival of the parasites was then determined over 4 days. Points represent the mean of two wells from two independent experiments±SEM. * Indicates significant difference $P \leq 0.05$ when compared to controls.

CHAPTER 7

THE EFFECTS OF GOLDFISH COMPLEMENT ON *T. danilewskyi IN VITRO*[2]

7.1 Introduction

The extracellular existence of *T. danilewskyi* predisposes it to direct contact with components of both the cellular and humoral branches of the immune system. Goldfish are highly susceptible to trypanosome infection, and although 60-80% mortality has been documented, fish that survive the infection become immune to homologous challenge [390, 403]. In addition, passive transfer studies have shown that immunity to re-infection can be transferred to naïve goldfish by injecting them with plasma or purified IgM from a recovered host [218, 283, 403]. Resistance to re-infection is long lasting (as long as 365 days) and is believed to be due to the sequestration of parasites in the internal organs resulting in continual priming of the immune system [170, 403]. Specific antibodies are generated towards *T. danilewskyi* during the course of infection and recently were shown to be induced in naïve goldfish after immunization with parasite excretory/secretory products [40, 43, 283]. Despite the demonstration of the presence of these specific antibodies in goldfish serum, nothing is known about how they may be acting to resolve the trypanosome infection.

One of the mechanisms in which antibodies may work to eliminate trypanosomes is by antibody-dependent complement-mediated lysis. The complement system in teleosts is very similar to that of mammals [66]. Complement plays a key role in the elimination of various disease-causing organisms in fish including blood-borne protozoans. For example, *Cryptobia salmositica* was lysed by serum from resistant, but not susceptible, laboratory-raised brook charr. The mechanism of innate resistance of brook charr was later attributed to complement-mediated lysis of parasites via the alternative complement pathway [120]. Studies using rainbow trout have shown that complement-fixing antibodies are produced against *C. salmositica* during live infection, or after vaccination with an attenuated strain of the parasite [108]. Specific antibodies have also been shown to bind to *T. borreli* in immunofluorescence studies. These antibodies were proposed to be involved in the lysis of the parasites by activation of the classical complement pathway [317].

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[2] Portions of this chapter have been published: Plouffe DA and Belosevic M. 2004. Enzyme treatment of *Trypanosoma danilewskyi* (Laveran & Mesnil) increases its susceptibility to lysis by the alternative complement pathway of goldfish (*Carassius auratus*) (L.). Journal of Fish Diseases. 27: 277-285.

The objective of the experiments described in this chapter was to determine the susceptibility of *in vitro* cultured *T. danilewskyi* to goldfish complement in the presence and absence of serum from recovered hosts, and if possible, to determine the route of complement activation by fish trypanosomes.

7.2 Experimental Design

7.2.1 Determination of the susceptibility of *in vitro* cultivated trypanosomes to lysis by serum from naïve and recovered hosts

To determine the susceptibility of *in vitro* cultured *T. danilewskyi* to goldfish complement, parasites were incubated with heat-inactivated or non-heat-inactivated (normal) serum from fish infected with *T. danilewskyi* (immune) or uninfected fish (non-immune). Non-immune fish serum was obtained from healthy goldfish while immune serum was obtained from goldfish that had been infected with 6.25×10^6 cultured trypanosomes. On days 0, 7, 14, 28, 42, and 56 post-infection whole blood was collected from 6 individual fish, the serum fractions were isolated, and equal volumes from each fish were pooled for use in experiments. Serum from infected fish has been previously shown to possess parasite specific antibodies and therefore is considered 'immune' [43, 283]. Heat inactivation of sera (56°C for 30 minutes) was required as a negative control for complement activity since the components of complement activation are heat-labile. Parasites were added to the various goldfish serum treatment groups in duplicate to the wells of a 96-well tissue culture plate and the plates were incubated for 1 hour at 20°C. Longer incubation times (16 hours) were also performed but did not change the susceptibility of the parasites to lysis. For wells containing immune serum, the effects of the presence of anti-parasite antibodies were determined by adding heat-inactivated immune serum in conjunction with normal serum from naïve hosts as a source of complement. Each experiment was repeated three times and the effect of exposure of the trypanosomes to the different sera was determined by direct enumeration of the remaining viable parasites using a hemocytometer. Each well was counted once.

7.2.2 Enzyme treatment of *in vitro* cultured trypanosomes

To determine whether parasite surface molecules, and in particular sialic acid residues, contributed to the resistance of *T. danilewskyi* to lysis, the parasites were treated with enzymes to strip proteins and sialic residues from the surface. Trypsin was employed as a general protease to remove surface proteins. Two different sialidase enzymes, one general (*Clostridium perfringens*) and one positionally specific (*Arthrobacter ureafaciens*; α-2→3,6,8,9), were used to remove sialic acid residues from the parasite surface glycoproteins. *In vitro* cultured trypanosomes were washed and re-suspended in serum-free culture medium. Enzyme solutions, or controls consisting of medium alone, were added to the trypanosome suspension and incubated for 30 minutes at either 37°C (sialidases) or 20°C (trypsin). Following the enzyme treatment, SBTI (trypsin treatment) or medium (sialidases) was added and the reaction continued for 10 minutes at either 20°C or 37°C. After inactivation of the enzymes, the parasites were washed and re-suspended for use in subsequent assays. An additional treatment involved the exposure of sialidase-treated parasites to normal and sialidase treated goldfish serum. This treatment was included to prevent the parasites from resialylating their surfaces using the *trans*-sialidase enzyme. This enzyme is unique to trypanosomes and is employed to transfer sialic acid residues from host glycoconjugates to the parasite surface proteins. Sialidase treated serum was prepared by incubating serum with *A. ureafaciens* sialidase or *C. perfringens* sialidase for 3 hours at 37°C. Enzyme treated parasites were exposed to normal and heat-inactivated serum from naïve and recovered hosts in duplicate wells of a 96-well plate. For some experiments the non-immune goldfish serum was diluted to determine the effects of dilution on the trypanolytic activity. The trypsin treated parasites were exposed to non-immune goldfish serum at 4°C and 20°C since the complement cascade of fish, in contrast to that of mammals, is active at low temperatures. Each experiment was performed at least three times and the effects of the treatments were determined by direct enumeration (one count/well) of the remaining parasites using a hemocytometer. Since the lysis of trypsin-treated *T. danilewskyi* occurred regardless of the presence of specific antibodies, non-immune serum was used in all subsequent assays.

7.2.3 Determination of the pathway(s) of complement activation

To determine which complement pathway is activated by trypsin-treated parasites, sera were treated with the chelating agents EGTA and EDTA to remove calcium and magnesium ions, which are required for the function of the lectin, and alternative cascades respectively. Prior to the addition of trypsin-treated, or untreated control trypanosomes, EDTA (ethylenediaminetetraacetic acid), or EGTA/Mg^{2+} (ethylenebis(oxyethylenenitrilo) tetraacetic acid+$MgCl_2$) was added to normal or heat inactivated non-immune goldfish serum. EGTA/Mg^{2+} was used to chelate calcium because EGTA may also non-specifically bind magnesium ions. Therefore, excess magnesium was added to prevent the removal of both ions in this treatment group. In some cases, sera were re-supplemented with ions prior to the addition of trypsin-treated parasites to ensure that the removal of ions was responsible for the result of the chelation treatment. The mean numbers of trypanosomes remaining following addition of the parasites to the chelated sera was calculated from duplicate wells of three replicate experiments.

7.2.4 Restoration of trypanosome resistance to complement-mediated lysis

To determine whether enzyme treated parasites could regain resistance to complement-mediated lysis, and to determine whether the ability to synthesize proteins is required for the development of resistance, treated parasites were returned to normal culture conditions (described in the materials and methods) in the presence or absence of puromycin. Puromycin is an antibiotic inhibitor of translation and would therefore inhibit the production of new proteins by the parasites. After 0, 6, and 24 hours of incubation under these conditions, samples of parasites from all treatments were removed and exposed to non-immune and heat-inactivated non-immune serum in duplicate wells of a 96-well culture plate. This experiment was replicated three times and the number of parasites remaining in each well following exposure to goldfish serum was determined by direct enumeration of the trypanosomes using a hemocytometer (one count/well).

7.3 Results

7.3.1 Susceptibility of normal and enzyme treated parasites to lysis by goldfish serum

Incubation of control (untreated) trypanosomes in non-immune goldfish serum, or serum obtained from infected goldfish (days 4 to 56 post infection), did not result in lysis of the parasites (Table 7-1). In contrast, treatment of trypanosomes with trypsin rendered parasites susceptible to lysis by non-immune serum and serum from goldfish with 4-56 day-old parasite infection because no viable parasites were observed following incubation (Table 7-1). Heat-inactivation of the serum abrogated this effect because trypsin treatment caused a minimal but significant reduction in parasite number (Table 7-1). Similar results were obtained when trypsin treated and control parasites were exposed to complete medium, non-immune serum, or heat-inactivated non-immune serum at 4°C (Table 7-2). No viable parasites remained following trypsin treatment and exposure to non-immune serum. There was no effect of the 4°C temperature incubation on parasite viability since there was no difference in the mean log numbers of treated and control parasites observed in complete medium following the low temperature incubation (Table 7-2).

When incubated in non-diluted non-immune serum, all trypsinized trypanosomes were killed. Treated trypanosomes were not susceptible to lysis when incubated in heat-inactivated serum, suggesting that a heat-labile component present in the serum was responsible for lysis. There was a dose-dependent decrease in the lysis of parasites exposed to decreasing concentrations non-immune serum (Figure 7-1).

Since non-immune serum and serum from trypanosome-infected fish killed trypsinized parasites, specific anti-parasite antibodies apparently were not required for lysis. Because similar results were obtained when both types of sera were used, undiluted non-immune goldfish serum was used in all subsequent experiments.

Treatment with sialidase enzymes had little or no effect on the number of trypanosomes remaining following exposure to goldfish serum. Pre-treatment of trypanosomes with *Arthrobacter ureafaciens* sialidase resulted in a significant increase in the mean numbers of trypanosomes although the absolute size of the increase was small (Table 7-3). The various serum types and treatments had a significant effect but, again, the magnitude of the effect was small. In a similar experiment, parasites were treated with *C. perfringens* sialidase for 30 minutes at 37°C. No effect on parasite numbers could be attributed to pre-treatment of parasites with sialidase, or to the five combinations of serum type and treatment (Table 7-4).

7.3.2 Effect of removal of divalent cations from goldfish serum on the susceptibility of trypsinized trypanosomes to lysis

Chelation of magnesium ions

Pre-treatment of non-immune goldfish serum with 10 mM EDTA completely prevented the killing of trypsinized trypanosomes. However, when pre-treated sera were re-supplemented with magnesium ions (80 mM $MgCl_2$), the ability of non-immune goldfish serum to lyse trypsinized parasites was fully restored (Table 7-5). The chelation reagents appeared to have little effect on the viability of the parasites. When trypanosomes were cultured in medium only, there was no effect of trypsin treatment but a significant effect of serum treatment. This effect was minimal because no significant differences could be detected in pair-wise comparisons.

Chelation of calcium ions

Pre-treatment of non-immune serum with 10 mM EGTA/8 mM Mg did not prevent the lysis of trypsin-treated trypanosomes (Table 7-6). Pre-treated sera re-supplemented with calcium ions (24 mM $CaCl_2$) retained the ability to cause lysis of treated parasites while having no effect on the survival of control trypanosomes. The reagents used did not affect parasite number. There was no significant effect of trypsin treatment or chelation treatment.

7.3.3 Restoration of resistance to lysis of trypsin-treated trypanosomes

Trypsinized trypanosomes were able to partially regain resistance to non-immune goldfish serum when incubated under normal culture conditions (medium + 10% heat-inactivated goldfish serum) for at least 6 hours (Figure 7-2). However, after 24 hours of incubation almost all of the trypanosomes were able to resist the lytic effect of serum. The incubation of parasites in the presence of puromycin (an antibiotic inhibitor of protein synthesis) abrogated the ability of the trypanosomes to regain resistance to lysis, suggesting that protein synthesis was required for re-acquisition of protection against the lytic effect of goldfish serum. The viability of trypanosomes was not affected by addition of puromycin since the numbers of those exposed to the drug were not significantly different from those that were not exposed to the drug for the heat-inactivated non-immune serum treatment.

7.4 Discussion

The results presented in this chapter suggest that untreated, cultured *T. danilewskyi* are not susceptible to complement-mediated lysis *in vitro* since there was no decrease in the numbers of parasites (not-enzyme treated) remaining in the wells following exposure to non-heat-inactivated immune and non-immune goldfish serum. This result seems to contradict the previous finding that IgM isolated from naïve and infected goldfish inhibits the growth of *T. danilewskyi in vitro* (Chapter 6). However, it is important to note that whole serum and not purified IgM was used for the complement studies. The inhibitory effects of IgM would likely not be observed in whole serum since the concentration of anti-trypanosome IgM would be less than when parasites were incubated with the purified goldfish immunoglobulin.

Many blood-borne pathogens including viruses, helminths, and protozoa have evolved ways by which they can prevent activation of the different complement pathways or prevent the completion of the lytic cascade [409]. Resistance to complement, a critical component of the innate host defense, would likely result in the rapid establishment of *T. danilewskyi* infection as well as the maintenance of chronic infections, both being characteristics of natural and experimental infections in fish [40, 216, 389].

Trypanosomes have acquired a variety of tools to escape eradication by complement. The mammalian trypanosomes, *T. brucei* and *T. cruzi*, are resistant to complement because they possess specialized surface coats. Bloodstream forms of fish trypanosomes possess a surface coat very similar to that of *T. cruzi* containing high copy numbers of mucin-like glycoproteins that are GPI-linked and are heavily modified by a variety of sugar moieties including sialic acid [214, 298]. The presence of sialic acid results in a negative surface charge that is thought to contribute to the resistance of the parasites to non-specific complement mediated lysis. The presence of polyanions, such as sialic acid, on the surface of cells is believed to stabilize and enhance the interaction of the host complement inhibitor factor H with the alternative pathway C3 convertase [243]. Treatment of *T. cruzi* with proteases and sialidases increases the susceptibility of the trypomastigotes to the alternative complement pathway; however, the level of susceptibility of desialylated parasites differs among studies. In some cases, 40-70% lysis of sialidase-treated parasites by the alternative complement pathway has been documented [195, 335]. However, other researchers utilizing a fluorescence-release assay to quantify parasite lysis have observed only 5% lysis of sialidase treated parasites. In the latter study, lysis of sialidase-treated parasites increased to 24% if the serum used as a source of complement was also treated with sialidase [366]. Reasons for the differences in susceptibility of sialidase-treated parasites to lysis include the source and form of parasites used, and the presence of proteases or azide in the enzyme preparations used in the former studies [366]. Both proteases and azide increase the susceptibility of *T. cruzi* to complement [195, 335, 366]. The results of the latter studies suggest that although the presence of sialic acid residues may enhance the deposition of the inactive form of C3b, iC3b, on normal parasites, it does not account for the complete resistance of trypomastigotes to complement-mediated lysis [366].

In the present study, *T. danilewskyi* were treated with proteolytic and glycolytic enzymes to determine whether this manipulation would increase the sensitivity of the parasites to non-immune goldfish serum, and to serum from fish that had been infected with trypanosomes. It was shown that the treatment of parasites with trypsin rendered them susceptible to lysis by both types of goldfish serum. Lysis did not occur when trypsin-treated trypanosomes were exposed to heat-inactivated serum suggesting that heat-labile serum components are required for lysis. In addition, lysis of parasites occurred at 4°C as well as 20°C and killing at low temperatures is a feature of the fish alternative complement pathway [66]. Since specific antibodies were not required for lysis of the parasites, it seemed likely that the alternative or lectin complement pathways are primarily responsible for killing of the trypsinized trypanosomes.

Because the fish complement pathways have the same requirement for divalent cations as their mammalian counterparts [66], Mg^{2+} and Ca^{2+} ions were selectively removed from the serum before addition of the treated parasites to identify which complement pathway was responsible for parasite lysis. The addition of EDTA completely blocked the lysis of parasites by non-immune goldfish serum. Re-supplementation of the EDTA-chelated serum with Mg^{2+}completely restored the lytic mechanism suggesting that magnesium was required for lysis. The removal of Ca^{2+} ions using EGTA/Mg did not block the lysis of treated trypanosomes supporting the hypothesis that lysis of treated trypanosomes occurs through the activity of the goldfish alternative complement pathway.

Treatment of *T. danilewskyi* with *A. ureafaciens* or *C. perfringens* sialidases did not enhance their susceptibility to complement from untreated non-immune goldfish serum. Since *T. danilewskyi*, like other trypanosomes, also possesses an active *trans*-sialidase enzyme [3] sialidase-treated parasites were exposed to sialidase-treated non-immune serum to prevent the resialylation of the parasites through the activity of this unique enzyme. Despite treatment of the serum with sialidases, the parasites remained resistant to lysis. Both sialidases (*C. perfringens* and *A. ureafaciens*) are effective in desialylating the glycoproteins of many cell types including *T. cruzi* trypomastigotes [195, 306, 335, 359]. Compositional analysis of the surface mucin-like proteins of fish trypanosomes suggests that they are modified by a more diverse array of sugar residues than are found on the *T. cruzi* surface carbohydrates, which could decrease the importance of a single sugar type [214]. Therefore, it is possible that modification of surface proteins with sialic acid is more important in protecting mammalian trypanosomes from complement mediated lysis than fish trypanosomes. However, as mentioned previously, the importance of sialic acid residues in contributing to the resistance of mammalian trypanosomes may have been overestimated [366].

T. cruzi trypomastigotes rendered susceptible to complement by enzyme treatment are able to regain resistance to host serum or plasma following incubation in culture for 6 hours [335]. Our findings also showed that trypsin-treated fish trypanosomes regain resistance to lysis by goldfish serum after at least 6 hours of incubation under normal culture conditions. To determine whether the restoration of resistance was related to the ability of the parasites to synthesize new surface proteins, an antibiotic inhibitor of translation, puromycin, was added to the culture medium. Parasites that were exposed to puromycin during culture were unable to regain resistance to the lytic effects of goldfish serum, even after 24 hours of incubation, while those that were not exposed to puromycin completely regained resistance to lysis after one day. Since neither puromycin nor trypsin had a detrimental effect on the viability of the trypanosomes, it is likely that protein synthesis was required for restoration of resistance to complement-mediated lysis of *T. danilewskyi*.

Currently, we are unable to determine whether there are specific proteins on the surface of *T. danilewskyi* that specifically bind and inactivate complement components, such as the complement regulatory protein found in *T. cruzi* [268]. An alternate explanation might lie in the nature of the surface coat of the parasites. The glycophosphatidylinositol (GPI)-anchored mucin-like proteins confer a net negative charge to the surface of the parasite and also provide a layer of protection for cryptic membrane proteins that may activate complement but are not normally exposed. Treatment of the parasites with a GPI-specific phospholipase C of parasite origin, or with glycolytic enzymes specific for the carbohydrate linkages found in the mucin-like molecules, would provide more information about the minimal molecular requirements for the resistance of *T. danilewskyi* to complement.

Table 7-1. Lysis of trypsin treated *Trypanosoma danilewskyi* by sera from infected and uninfected goldfish.

Serum Type: Days post infection	Parasite Treatment	
	Control	Trypsinized
HI non-immune	5.4± 011	5.2± 0.12 *
Non-immune	5.4±0.15	0.0± 0.00 *
Immune:		
4	5.4± 0.09	0.0± 0.00 *
14	5.5± 0.11	0.0± 0.00 *
28	5.4± 0.07	0.0± 0.00 *
42	5.5± 0.07	0.0± 0.00 *
56	5.4±0.08	0.0± 0.00 *

Control and trypsin-treated cultured trypanosomes were exposed to serum from both infected and uninfected goldfish as described in the methods for 1 hour at 20°C. Data are mean numbers of parasites per well ± SEM ($\log_{10}x+1$), calculated from duplicate wells of three replicate experiments. (*) indicates significant difference ($P \leq 0.05$) in Bonferroni-corrected pair-wise comparisons between trypsinized parasites and corresponding controls. HI denotes heat-inactivated non-immune serum. Immune refers to pooled serum collected from 6 individual fish infected with *T. danilewskyi* after the number of days indicated.

Table 7-2. Lysis of trypsin treated *Trypanosoma danilewskyi* by non-immune goldfish serum at low temperature.

Serum Type	Parasite treatment	
	Control	Trypsinized
Medium	4.9±0.05	4.9±0.02
HI non-immune	4.8±0.04	4.9±0.03
Non-immune	4.8±0.06	0.0±0.00 *

Control and trypsin-treated cultured trypanosomes were exposed to serum from uninfected goldfish as described in the methods for 1 hour at 4°C. Data are mean numbers of parasites per well ± SEM ($\log_{10}x+1$) calculated from duplicate wells of three replicate experiments. (*) indicates significant difference ($P\leq0.05$) in Bonferroni-corrected pair-wise comparisons with all other values in the table. HI denotes heat-inactivated non-immune serum. Medium refers to trypanosome culture medium including 10% heat-inactivated goldfish serum.

Table 7-3. The effect of *Arthrobacter ureafaciens* sialidase treatment on the susceptibility of *Trypanosoma danilewskyi* to lysis by normal and sialidase-treated non-immune goldfish serum.

Serum Type	Parasite treatment	
	Control	Sialidase
Medium only	4.8±0.03	4.9±0.02
HI non-immune	4.8±0.04^{NS}	4.9±0.15 *
Non-immune	4.9±0.01^{NS}	4.9±0.10 *
HI non-immune + sialidase	4.9±0.01^{NS}	5.0±0.09 *
Non-immune + sialidase	4.8±0.10^{NS}	4.8±0.01^{NS}

Control and *A. ureafaciens* sialidase-treated trypanosomes were exposed to normal or *A. ureafaciens* treated non-immune goldfish serum for 1 hour at 20°C. Data are mean numbers of parasites per well ± SEM ($\log_{10}x+1$) calculated from duplicate wells of three replicate experiments. (*) indicates significant difference ($P \leq 0.05$) and (NS) designates no significant difference when compared to control medium only. HI denotes heat-inactivated non-immune serum. Medium refers to trypanosome culture medium including 10% heat-inactivated goldfish serum.

Table 7-4. The effect of *Clostridium perfringens* sialidase treatment on the susceptibility of *Trypanosoma danilewskyi* to lysis by normal and sialidase-treated non-immune goldfish serum.

Serum Type	Parasite treatment	
	Control	Sialidase
Medium only	4.8±0.07	4.9±0.01
HI non-immune	4.8±0.06	4.8±0.05
Non-immune	4.8±0.05	4.8±0.04
HI non-immune + sialidase	4.8±0.07	4.8±0.08
Non-immune + sialidase	4.8±0.08	4.9±0.04

Control and *C. perfringens* sialidase treated trypanosomes were exposed to normal or *C. perfringens* treated non-immune goldfish serum for 1 hour at 20°C. Data are mean numbers of parasites per well ± SEM ($\log_{10}x+1$) calculated from duplicate wells of three replicate experiments. No significant difference was found between any treatment groups. HI denotes heat-inactivated non-immune serum. Medium refers to trypanosome culture medium including 10% heat-inactivated goldfish serum.

Table 7-5. The effects of Mg^{2+} chelation on the ability of non-immune goldfish serum to cause lysis of trypsin treated *Trypanosoma danilewskyi*.

Parasite Treatment	Serum Type	Serum treatment			
		None	+ EDTA	+ Mg^{2+}	+ EDTA & Mg^{2+}
Control					
	Medium	5.0±0.13	4.9±0.08	4.9±0.06	4.9±0.08
	HI-non-immune	5.7±0.03	5.5±0.04	ND	5.5±0.07
	Non-immune	5.6±0.04	5.6±0.02	ND	5.6±0.04
Trypsin					
	Medium	5.1±0.16	4.8±0.10	4.8±0.10	4.8±0.15
	HI-non-immune	5.5±0.04	5.5±0.03	ND	5.3±0.07
	Non-immune	0.0±0.00 *	5.5±0.06	ND	0.0±0.00 *

Control and trypsin treated cultured trypanosomes were exposed to normal and heat-inactivated non-immune goldfish serum as described in the methods for 1 hour at 20^0C. In a number of wells magnesium ions were chelated with 0.01 M EDTA and in some wells containing chelated sera, magnesium ions were restored using 0.08M $MgCl_2$. Data are mean numbers of parasites per well ± SEM ($\log_{10}x+1$) calculated from duplicate wells of three replicate experiments. (*) indicates significant difference ($P \leq 0.05$) in Bonferroni-corrected pair-wise comparisons with all other values in the same column.. HI denotes heat-inactivated non-immune serum and ND denotes not done. Medium refers to trypanosome culture medium including 10% heat-inactivated goldfish serum.

Table 7-6. The effect of Ca^{2+} chelation on the ability of non-immune goldfish serum to cause lysis of trypsin treated *Trypanosoma danilewskyi*.

		Serum treatment			
Parasite Treatment	Serum Type	None	+EGTA/Mg^{2+}	+ Ca^{2+}	+EGTA/Mg + Ca^{2+}
Control					
	Medium	4.7±0.08	4.6±0.09	4.6±0.05	4.7±0.10
	HI-non-immune	4.8±0.07	4.7±0.10	ND	4.6±0.01
	Non-immune	4.7±0.08	4.7±0.01	ND	4.7±0.11
Trypsin					
	Medium	4.6±0.04	4.6±0.05	4.6±0.04	4.6±0.07
	HI-non-immune	4.7±0.08	4.7±0.07	ND	4.7±0.05
	Non-immune	0.0±0.00 *	0.0±0.00 *	ND	0.0±0.00 *

Control and trypsin-treated cultured trypanosomes were exposed to normal and heat-inactivated non-immune goldfish serum as described in the methods for 1 hour at 20^0C. In a number of wells calcium ions were chelated with 0.01 M EGTA/Mg^{2+} and in some wells containing chelated sera, calcium ions were restored using 0.024 M $MgCl_2$. Data are mean numbers of parasites per well ± SEM ($\log_{10}x+1$) calculated from duplicate wells of three replicate experiments. (*) indicates significant difference ($P \leq 0.05$) in Bonferroni-corrected pair-wise comparisons with all other values in the same column. HI denotes heat-inactivated non-immune serum and ND denotes not done. Medium refers to trypanosome culture medium including 10% heat-inactivated goldfish serum.

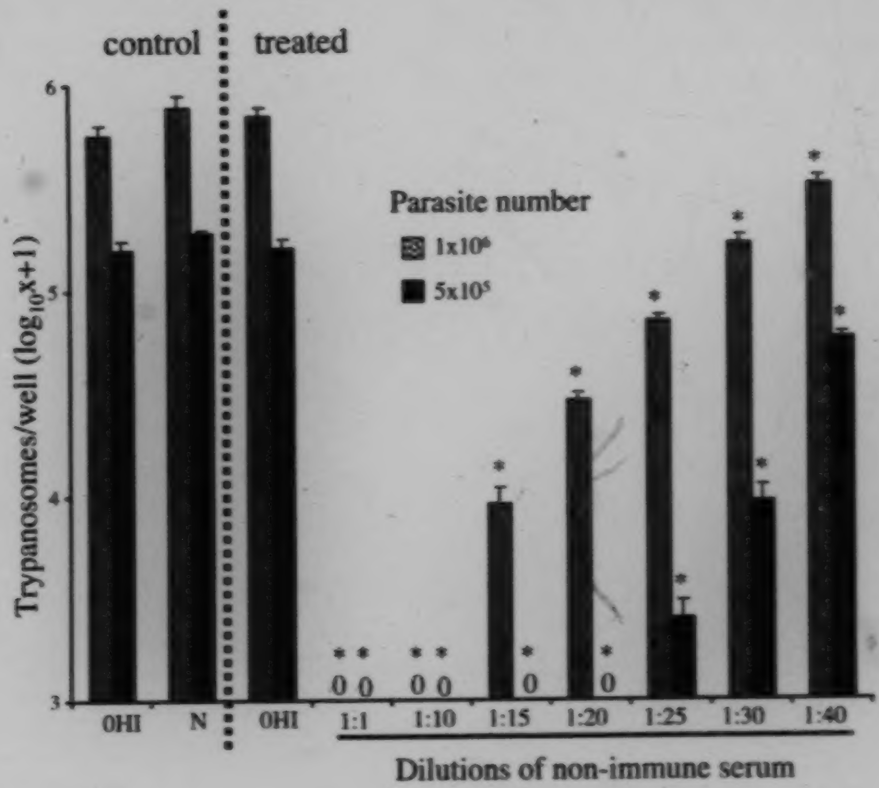

Figure 7-1. Effect of serum dilution on lysis of two different concentrations of trypanosomes. 1×10^6 or 1×10^5 untreated parasites were exposed to either undiluted heat-inactivated non-immune serum (0HI), or undiluted non-immune serum (0). The same two concentrations of trypsinized trypanosomes were incubated with undiluted heat-inactivated serum (0HI) and non-immune serum (N) as well as various dilutions of non-immune serum (1:10-1:40). Incubations were performed for one hour at 20^0C. Bars represent mean numbers of parasites per well ± SEM ($\log_{10}x+1$) calculated from duplicate wells of three replicate experiments. (*) indicates significant difference ($P\leq0.05$) in numbers of trypsinized parasites compared to the corresponding number in heat-inactivated non-immune serum treatment. Numbers of control parasites did not differ significantly from treated parasites in 0HI.

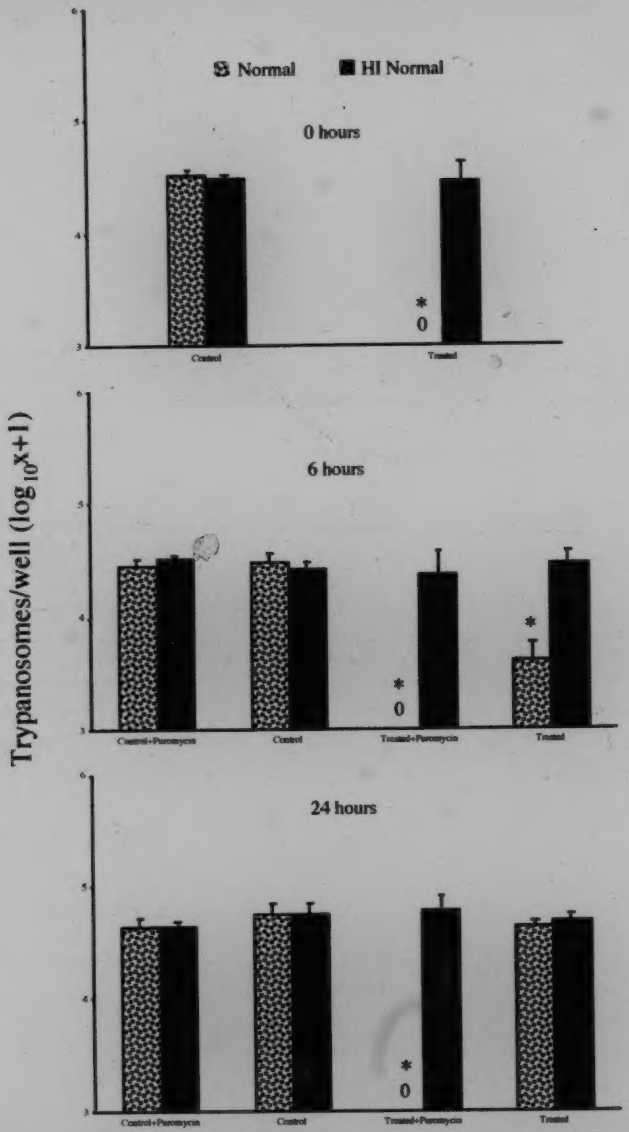

Figure 7-2. Ability of trypsinized trypanosomes to regain resistance to lysis by goldfish serum. Enzyme treated and untreated (control) parasites were incubated under normal culture conditions (medium+10% heat-inactivated goldfish serum) for 0, 6, and 24 hours in the presence or absence of 10 μg/ml puromycin. Following each time period treated and control parasites were exposed to either normal or heat-inactivated non-immune goldfish serum for 1 hour at 20°C. Bars represent mean numbers of parasites per well ± SEM ($\log_{10}x+1$) calculated from duplicate wells of three replicate experiments. (*) indicates significant difference ($P \leq 0.05$) in Bonferroni-corrected pair-wise comparisons between trypsinized parasites and the corresponding heat-inactivated non-immune serum treatment. All other pair-wise comparisons between adjacent bars or, for control versus control+puromycin treatments for similar sera, were not significant.

CHAPTER 8

PURIFICATION OF GOLDFISH COMPLEMENT COMPONENT THREE (C3) IN AN ATTEMPT TO IDENTIFY A *T. danilewskyi* COMPLEMENT-BINDING PROTEIN

8.1 Introduction

Complement component three (C3) is the central molecule in the complement cascade. It participates in the labeling of target molecules and cells for elimination, and its breakdown products are involved in regulation of the acquired immune response. C3 is a 190 kDa serum glycoprotein consisting of two disulfide-linked chains, the α chain (115 kDa), and the β chain (75 kDa). C3 is a member of the family of thioester-containing proteins that includes complement components 4 and 5 (C4 and C5) as well as α-2-macroglobulin, a serum protease inhibitor. Homology between these molecules suggests that they have evolved from a common ancestor [322]. With the exception of C5, all of the members of this family contain a unique, highly reactive, intra-chain thioester bond in the α-chain [205]. Proteolytic cleavage of C3 results in production of two fragments, a smaller molecule, C3a, and a larger fragment, C3b [206]. C3b contains the exposed thioester site that binds covalently to the target surface by hydrolysis of the thioester bond. Cleavage and binding of C3 is catalyzed by a histidine residue 100 amino acids C-terminal of the thioester site and by a glutamic acid residue two amino acids downstream of the catalytic histidine [322]. If the exposed thioester site is not immediately bound it is rapidly hydrolyzed and inactivated to prevent promiscuous binding of C3b to non-target surfaces.

C3 is continually susceptible to the activity of the alternative pathway of complement activation. In mammals, host cells are protected from non-specific lysis by complement regulatory molecules such as factor H, and decay accelerating factor (DAF) [206]. In addition, modification of host cell surface glycoproteins with polyanionic molecules, including sialic acid, facilitates the activity of some complement regulatory proteins. In this manner, cell surfaces that are non-activating, such as those that are of host origin, potentiate the breakdown of deposited C3b into its inactive form, iC3b [243]. iC3b cannot participate in formation of the C3 convertase leading to target cell lysis but it is an important ligand for receptors that are present on phagocytic cells. Furthermore, it can also be degraded into C3dg and C3d, which are important for the generation of an efficient adaptive immune response [82, 125, 206].

A number of different blood-borne parasites have evolved specific complement binding molecules that perform functions similar to those of the host's own regulatory molecules, and many bear resemblance to the host molecules at the gene and protein level [163, 291]. In addition, many organisms are able to incorporate host complement regulatory molecules into their own cell membranes where they protect the pathogen from complement-mediated lysis [10, 244, 409]. Other strategies employed by pathogens to prevent complement activity include the production of proteases that degrade complement components [88, 100, 187, 307], and the presentation of non-activating surfaces rich in sialic acid residues [231, 366, 395]. *T. cruzi* uses a number of techniques to avoid complement-mediated lysis. The surface coat of *T. cruzi* consists of multiple copies of GPI-anchored mucin-like glycoproteins that are regularly modified with sialic acid residues [56, 85]. Trypanosomes are unable to synthesize these carbohydrates on their own and therefore employ a unique enzyme known as the *trans*-sialidase to transfer the molecules from the host glycoconjugates to the parasite surface [70]. While removal of the sialic acid moieties from the surface of *T. cruzi* has been shown to moderately increase susceptibility of the parasites to complement, the trypanosomes also possess a specific molecule known as the complement regulatory protein (CRP). The CRP is believed to be responsible for the majority of the resistance of the bloodstream forms to complement activity [366].

T. danilewskyi is also resistant to complement-mediated lysis by goldfish serum in *in vitro* assays [296]. Fish trypanosomes also possess a surface coat rich in mucin-like glycoproteins that are modified by sialic acid residues [214]. However, removal of the sialic acid residues using two different sialidase enzymes does not increase the susceptibility of the parasites to lysis. In addition, since the ability to synthesize and replace trypsin-cleaved surface proteins is a requirement for resistance to complement, it was hypothesized that *T. danilewskyi* may also possess a specific complement-binding molecule [296].

This chapter describes the purification of goldfish C3 that was undertaken in order to isolate a complement binding protein from *T. danilewskyi* that may be responsible for conferring resistance to lysis by goldfish complement. In addition, a number of different molecular and biochemical techniques were employed to determine whether a CRP homologue could be identified from *T. danilewskyi*.

8.2 Experimental Design

Two approaches were taken in order to identify a *T. danilewskyi* complement binding protein that might be responsible for resistance of the parasite to goldfish complement *in vitro*.

The first approach involved purification of goldfish C3 that was cleaved into C3a and C3b using trypsin. C3b was then coupled to an agarose matrix and used to isolate the potential native binding protein from parasite whole cell lysates. This approach was chosen because C3b affinity chromatography was used to purify the *T. cruzi* CRP [268]. The purified, cleaved C3 was also used in Far-Western blotting to probe parasite whole cell lysates, trypsin-cleaved surface proteins, and detergent soluble membrane extracts for the presence of a molecule that would bind goldfish C3b.

The second approach involved attempting to determine whether *T. danilewskyi* possesses a homologue of the *T. cruzi* CRP. To do this, RT-PCR using primers designed to the *T. cruzi* CRP gene sequence, as well as the screening of a *T. danilewskyi* cDNA library developed in our lab, were performed to isolate a gene similar to that encoding the *T. cruzi* CRP.

8.2.1 Purification of goldfish C3

Goldfish C3 was purified from the pooled serum of healthy fish in a manner similar to the procedures outlined by Nakao *et al.* [255] for the purification of carp C3. The general isolation procedure involved PEG precipitation of goldfish serum followed by anion exchange on a fast flow Q-sepharose column, size separation using a superose 6 column, and finally, anion exchange using a Mono-Q column. The identity and purity of the goldfish C3 following each step was determined by immunoblotting and SDS-PAGE. A rabbit-anti-carp C3 antibody obtained from Dr. M. Nakao that cross-reacted with goldfish C3 (see materials and methods in Chapter 3) was used for Western blotting. The identity of the purified molecule was confirmed by mass spectrometry. The fractions containing the purified C3 were sent to the UVic-Genome BC Proteomics Center located on the University of Victoria campus (www.proteincentre.com). Results of Q-TOF-Nanospray-tandem mass spectrometry (PE SCIEX API Pulsar) and peptide mapping of trypsin-digested samples were compared to a database of known proteins.

8.2.1.1 C3b affinity chromatography

Purified goldfish C3 was cleaved using trypsin and coupled to a cyanogen bromide (CnBr)-activated column as described for the purification of *T. cruzi* CRP [268]. *T. danilewskyi* lysates equivalent to 8×10^8 parasites were applied to the column and eluted in 1 mL fractions using glycine buffer (pH 2.5). The elution fractions were collected and analyzed by gel electrophoresis and silver staining.

8.2.1.2 Far-Western blotting

The purified, trypsin-cleaved goldfish C3 was also used to probe *T. danilewskyi* whole cell lysates, integral membrane proteins (stripped from the membrane using Triton X-114), and molecules found in the supernatant following trypsin-treatment of parasites (as described in materials and methods, Chapter 3). These parasite extracts were separated by gel electrophoresis under reducing, non-reducing, and native conditions, transferred to nitrocellulose, and probed with the cleaved goldfish C3, and affinity purified rabbit-anti-goldfish C3 IgG prepared in our lab.

8.2.2 RT-PCR using primers designed from the *T. cruzi* CRP and screening of a *T. danilewskyi* cDNA library

Two sets of primers were designed to unique portions of the *T. cruzi* CRP gene (Table 3-2). The *T. cruzi* CRP is a member of a large gene family that also includes the *T. cruzi trans*-sialidase. The members of this family share a number of sequence similarities including the presence of the sialidase domain [2]. Therefore, there are a limited number of regions of unique sequence for *T. cruzi* CRP, and a restricted number of primer pairs that could be designed for detection of a similar gene sequence in *T. danilewskyi*. Since *T. danilewskyi* also possesses a gene encoding a *trans*-sialidase, it was theorized that a probe could be generated from the sialidase domain of this sequence and used to probe a *T. danilewskyi* cDNA library that had been previously constructed.

8.3 Results

8.3.1 Purification of goldfish C3 from serum

Western blotting of the PEG precipitated goldfish serum revealed the presence of the C3 α (115 kDa) and β (70-75 kDa) chains in the 15% and 26% pellets. There was also some C3 that was not precipitated and remained in the supernatant (Figure 8-1). The 15% and 26% pellets were combined and applied to a Q-sepharose anion exchange column. The majority of the goldfish C3 was eluted following the application of the 100-300 mM NaCl elution buffers. All of the C3 was bound to the column prior to the first elution (50 mM) since none was recovered in the wash fraction (Figure 8-2). The fractions from the Q-sepharose column were pooled and applied to a superose 6 size-separation column. Fractions were eluted in 1X PBS and the fractions containing the semi-purified goldfish C3 (24-27) were pooled, and concentrated (Figure 8-3b). Since fractions 24-27 still contained some contaminating molecules as determined by silver staining, the pooled, concentrated fractions were further fractionated by anion exchange using a Mono-Q column. The final anion exchange step resulted in the isolation of purified goldfish C3 in fractions 17 and 18 (Figure 8-4). The identity of isolated proteins as C3 was confirmed by mass spectrometry as described in the materials and methods. It was determined that the peptides generated from trypsinization of the sample had similarity to carp C3 proteins.

8.3.2 C3b affinity chromatography

Despite the application of a large concentration of parasite products to the C3b-affinity column, no protein bands were detected in any of the elution fractions. This result indicates that the parasite lysates did not contain a molecule that could bind to the goldfish C3b immobilized on the column.

8.3.3 Far-Western blotting

T. danilewskyi whole cell lysates, detergent-soluble membrane proteins, and the supernatants from trypsin treated parasites were separated using gel electrophoresis under reducing, non-reducing, and native conditions. The size-separated molecules were subsequently transferred to nitrocellulose. No protein bands were identified in any of these parasite preparations following exposure to trypsin-treated goldfish C3 and incubation of the blots with affinity purified rabbit-anti-goldfish C3 IgG.

8.3.4 RT-PCR using primers designed from the *T. cruzi* CRP and screening of a *T. danilewskyi* cDNA library

There were no PCR products amplified from *T. danilewskyi* cDNA using the primer sets that were designed from the *T. cruzi* complement regulatory protein. A probe was generated by amplification of the sequence encoding the sialidase domain of the *T. danilewskyi trans*-sialidase molecule using RT-PCR (primer sequences in Table 3-2); however, a screen of greater than $5x10^5$ clones did not result in the identification of any genes that might be involved in complement resistance. While more clones from the cDNA library could be screened, it is unlikely that a CRP homologue would be identified using this technique. As mentioned previously, the *T. cruzi* CRP is a member of family of greater than 800 genes, some of which have also been identified in *T. danilewskyi* [3, 84]. Therefore, it is possible that a family of related genes of similar size also exists for *T. danilewskyi*. Therefore, the identification of any one member of such a family from a cDNA library would be very difficult.

8.4 Discussion

In this chapter the purification of goldfish C3 by PEG precipitation, anion exchange chromatography, and size separation chromatography was described. In addition, several unsuccessful attempts to identify a *T. danilewskyi* binding protein were outlined. The purification of goldfish C3 was performed to obtain material for the detection of a *T. danilewskyi* complement binding protein that may be involved in the resistance of the parasites to complement-mediated lysis. Teleost C3 is similar to mammalian C3 in that it consists of an α (115 kDa) and a β chain (75 kDa) that are linked by disulfide bonds. The purified goldfish C3 was used to generate a C3b affinity column, and for Far-Western blotting. A trypanosome molecule that binds C3b could not be identified in parasite whole cell lysates, trypsin-cleaved surface molecules, or integral membrane proteins using these techniques. While the results of these experiments do not rule out the existence of such a molecule, they do suggest the possibility that some other parasite surface molecule that does not bind C3b might be involved in resistance to complement. These possibilities include (1) the presence of other polyanionic molecules on the surface that enhance the activity of host complement regulatory molecules such as factor H (factor H-like activity has been identified in teleosts [193]); (2) the presence of a membrane bound protease, similar to gp63 of *Leishmania* and *T. brucei*, that inactivates complement components; and (3) steric hindrance of binding of the goldfish complement components imposed by the glycoprotein surface coat of the parasites.

Proteases with anti-complement activity include the major surface metalloprotease, gp63, of *Leishmania*, and possibly the gp63-like molecules of *T. brucei* [53, 88]. In *Leishmania*, the activity of gp63 results in the conversion of C3b bound to the parasite surface to iC3b, and reduces the fixation of the terminal complement components. Although iC3b does not activate the lytic cascade it does bind to complement receptors and promotes phagocytosis of the parasites by macrophages [53]. The fish hemoflagellate *C. salmositica* possesses a metalloprotease that contributes to the virulence of the parasites but has not been specifically implicated in the inactivation of host complement components [418]. To determine whether either of these mechanisms are responsible for the resistance of *T. danilewskyi* to complement, the proteolytic activity of *T. danilewskyi* lysates and supernatants could be tested for the ability to cleave C3 to its inactive form iC3b. This strategy would require a monoclonal antibody to goldfish C3, or antibodies that would recognize the terminal components of the lytic cascade of goldfish. In addition, glycolytic enzymes that are specific for the *O*-linked carbohydrate side chains of trypanosome mucin-like glycoproteins, or a trypanosome-derived GPI-specific phospholipase C enzyme, could be used to remove the entire GPI-anchored surface coat (or at least the carbohydrate modifications). The susceptibility of the parasites to goldfish complement following these enzyme treatments could then be determined as previously described [296].

The *T. cruzi* CRP is a developmentally regulated molecule that is found only on the surface of the bloodstream forms of the parasite. The molecule has been reported to share similar structure and function with the human complement regulatory molecule DAF [268]. The epimastigote stage of *T. cruzi* that develops in the vector is normally susceptible to complement-mediated lysis by mammalian serum; however, transfection with the CRP gene renders the insect stage resistant to lysis [267]. This result indicates clearly the importance of this molecule for maintaining trypanosome infection in the vertebrate host. Binding of the CRP to C3b also increases the susceptibility of the CRP GPI-anchor to cleavage by parasite specific phospholipases, and as a result the CRP-C3b complexes are released from the parasite surface [266]. A soluble form of the CRP could be involved in binding complement components away from the surface of the parasite, ensuring the prevention of C3b deposition on the surface of the trypanosomes. Antibodies that are generated towards the *T. cruzi* CRP late in the infection can prevent its activity, and render the trypomastigotes susceptible to complement-mediated lysis by the alternative complement pathway. Likewise, immunization of mice with CRP DNA induces a protective immune response and production of antibodies that are capable of lysing parasites in the presence of complement [330]. Therefore, the *T. cruzi* CRP is a virulence factor that is critical for the establishment of the trypanosomes, and targeting of the molecule by the host immune system can result in the resolution of the infection.

It was hypothesized that *T. danilewskyi* may possess a CRP homologue since trypsinization of the parasites renders them susceptible to complement-mediated lysis by the alternative pathway of complement activation. Following RT-PCR and screening of a *T. danilewskyi* cDNA library, a *T. danilewskyi* gene similar to that encoding *T. cruzi* CRP was not identified. The size of the gene family encoding the CRP in *T. cruzi*, and the similarity in sequence between its members makes it difficult to identify a similar molecule in *T. danilewskyi* using molecular techniques. However, these findings support the results of the goldfish C3b-affinty chromatography and Far-Western blotting, which suggest that *T. danilewskyi* does not possess a specific C3b-binding molecule similar to *T. cruzi* CRP. Confirmation of these results could be obtained by probing the *T. danilewskyi* cDNA library with a CRP probe generated from *T. cruzi* cDNA.

Like *T. cruzi*, the resistance of *T. danilewskyi* to lysis by complement is likely multi-factorial. Regardless of the nature of the *T. danilewskyi* surface molecules that protect the parasites from complement mediated lysis, neutralizing antibodies produced by the host could play a role in resolving the infection. Antibodies binding to soluble and cell surface glycoconjugates, complement binding proteins, or proteases would inhibit their activity and increase the susceptibility of the parasites to the effects of complement including target cell lysis, the enhancement of phagocytosis, and the generation of an effective acquired immune response.

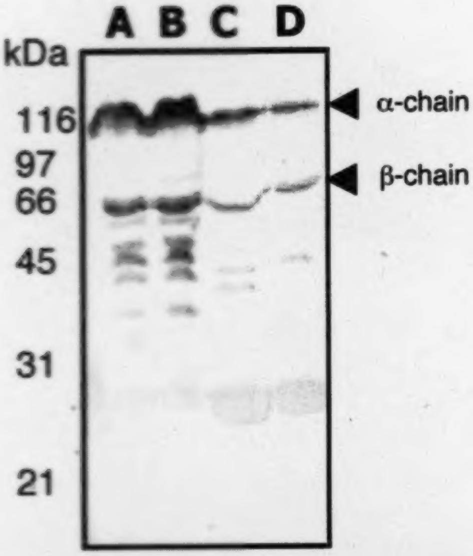

Figure 8-1 Polyethyleneglycol precipitation of goldfish serum for isolation of goldfish complement component three (C3) , Goldfish serum was precipitated with 15% and 26% polyethyleneglycol 3500. The resuspended pellets from the 15% (A) and 26% (B) precipitations as well as the combined pellets (C) and combined supernatants (D) were analyzed for the presence of goldfish C3 by Western blotting with rabbit-anti-carp C3 diluted 1:1000 in blocking buffer.

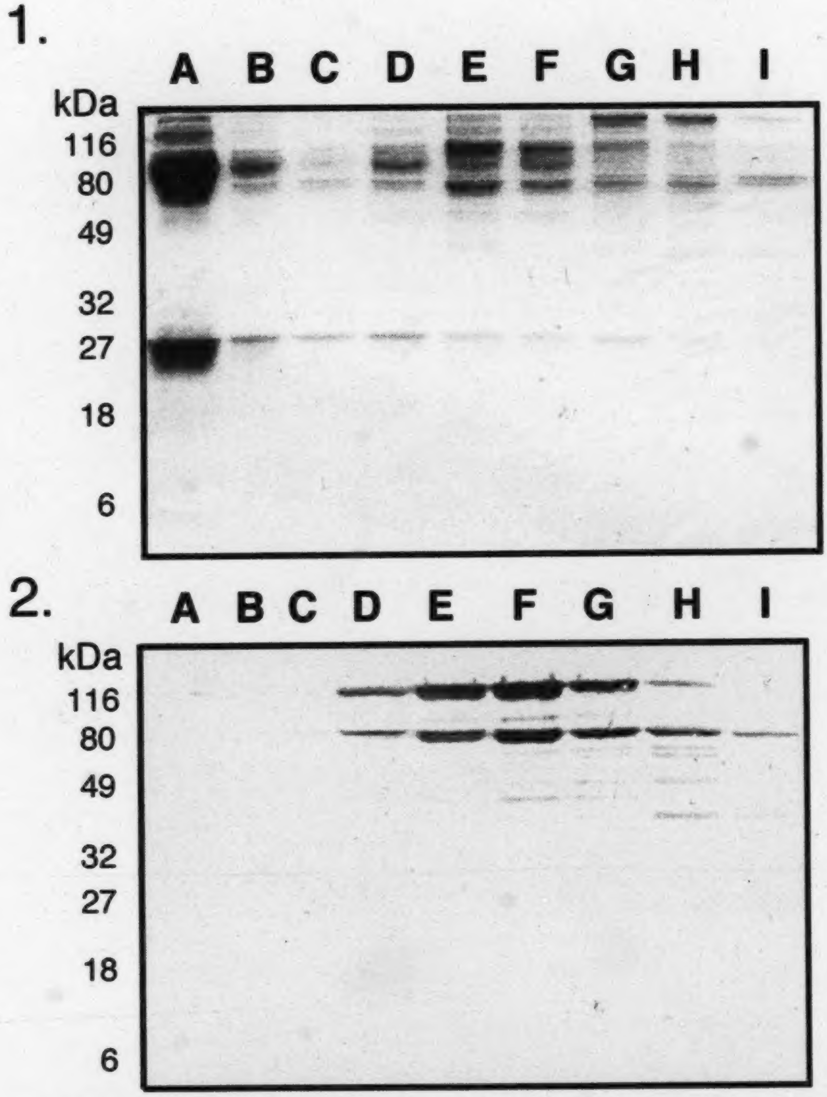

Figure 8-2. Polyethyleneglycol precipitated goldfish C3 separated by anion exchange chromatography. The resuspended, polyethylene glycol precipitated pellet containing goldfish C3 was applied to a Q-sepharose fast flow column (HiTrap Q-FF), the column was washed and subsequently eluted with a step-wise gradient of NaCl (50 mM-300 mM). Following elution, the column was regenerated using wash buffer+1M NaCl. Samples of the fractions collected following application (A), wash (B), elutions with 50 mM (C), 100 mM (D), 150 mM (E), 200 mM (F), 250 mM (G), 300 mM (H) NaCl, and regeneration (I) were analyzed for the presence of goldfish C3 using gel electrophoresis (1) and immunoblotting with rabbit-anti-carp C3 diluted 1:1000 in blocking buffer (2).

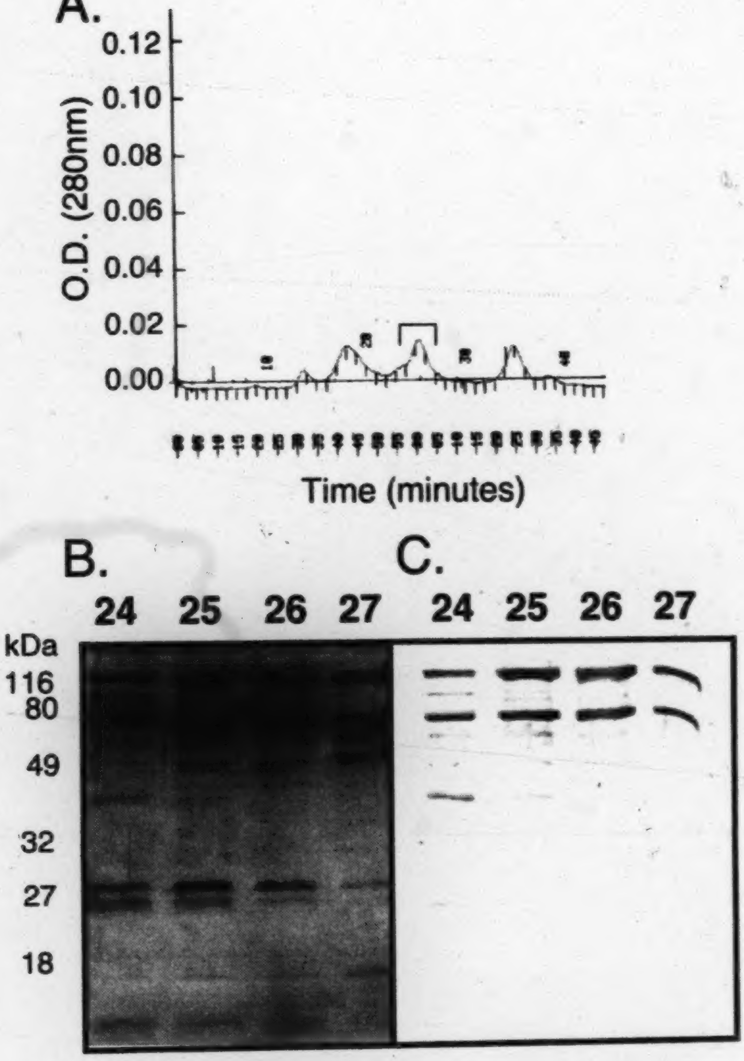

Figure 8-3. Gel permeation fast performance liquid chromatography (GP-FPLC) separation of the goldfish C3-containing fractions isolated by anion exchange using the HiTrap Q-FF column. Proteins were separated based on size using a Superose 6 column (A). Fractions were eluted in resuspension buffer at a flow rate of 0.4 mL/minute and collected in 15 mL centrifuge tubes. Semi-purified Goldfish C3 was identified in fractions 24-27 by gel electrophoresis (silver staining) (B) and Western blotting with rabbit-anti-carp C3 diluted 1:1000 in blocking buffer (C).

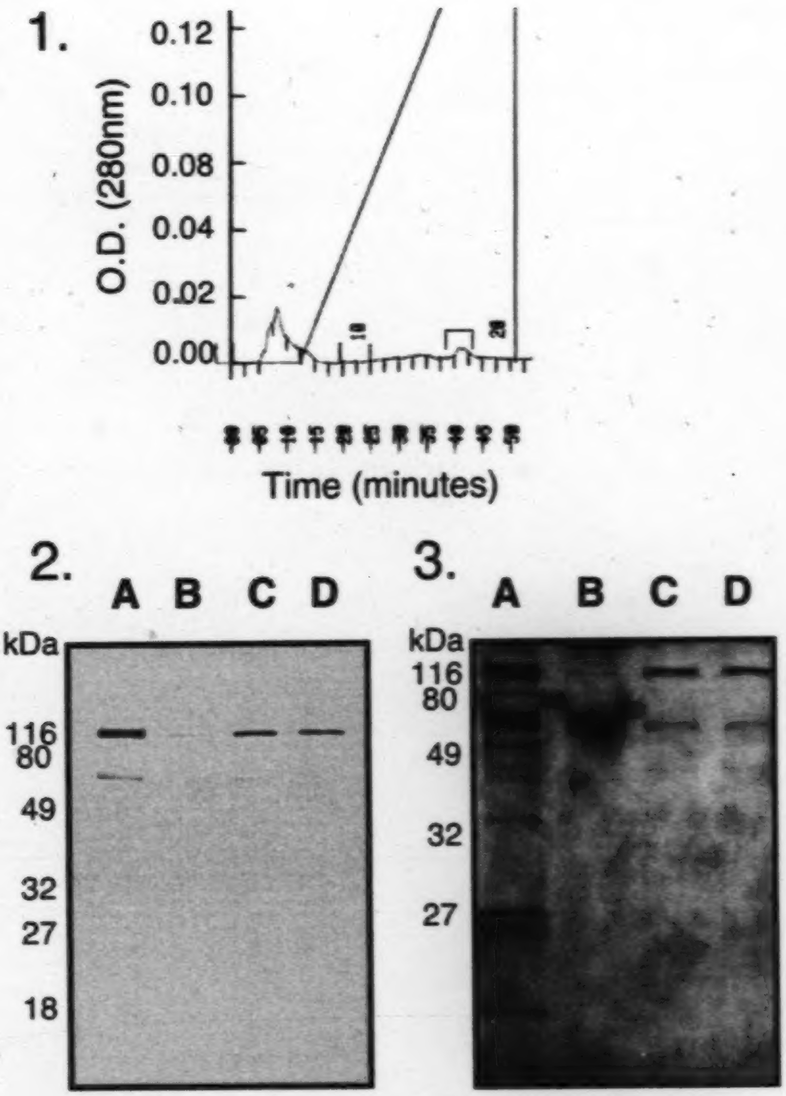

Figure 8-4. Final purification of goldfish C3 by anion exchange using a Mono-Q column. Fractions 24-27 from the Superose 6 column were pooled, concentrated and applied in multiple injections to the Mono-Q column. Proteins were eluted into 15 mL centrifuge tubes using a linear ascending salt gradient at a flow rate of 0.75 mL/minute (1). Purified goldfish C3 was detected by immunoblotting with rabbit-anti-carp C3 diluted 1:1000 in blocking buffer in the applied sample (2A) and fractions 17 and 18 (2C&D) but not in the unbound fraction (2B). Purity of the samples was determined by silver staining of the applied sample (3A), the unbound fraction (3B), and fractions 17 and 18 (3C&D).

CHAPTER 9

GENERAL DISCUSSION

Trypanosoma danilewskyi infections represent an excellent model system for understanding the mechanisms of immunity in fishes, and the nature of trypanosome infections in lower vertebrates. Despite the high prevalence of trypanosome infections in fish and other aquatic vertebrates, these species of parasites are generally considered to be non-pathogenic. The morbidity and mortality that has been associated with trypanosomiasis in some aquaculture settings suggest that the apparent lack of pathogenicity in fish is an immunological phenomenon related to the ability of healthy fish to control the infection [283]. The overall objective of the studies described in this thesis was to further characterize the immune response of fish to *T. danilewskyi* by examining the role of complement and parasite-specific antibodies in mediating protective immunity in goldfish.

9.1 Overview of findings

Previously we have shown that immunization of goldfish with the excretory-secretory (ES) products of *T. danilewskyi* increases their resistance to secondary infection [43]. The first objective of my thesis work was to identify specific component(s) of trypanosome ES products that induce protective immunity. Western blotting of fractionated ES products with serum that was pooled from recovered fish, and mass spectrometry were used to identify antigenic proteins that had high amino acid sequence similarity to α- and β-tubulin from *T. cruzi*. Although this result was unexpected, and although tubulin is most certainly not the only parasite antigen that is immunogenic in goldfish, additional studies have shown that trypanosome tubulin can be found in the extracellular environment, and that it can also induce a protective immune responses in mammalian models of trypanosome infection [36, 220].

The α- and β-tubulin protein-coding sequences were determined for *T. danilewskyi*, and recombinant molecules were successfully expressed in *E. coli*. Alignment of the predicted amino acid sequences for *T. danilewskyi* α- and β-tubulin revealed a high degree of similarity to corresponding sequences of other flagellated protozoans. In contrast, the degree of conservation between the *T. danilewskyi* α- and β-tubulin sequences and those of a number of vertebrate species was not as high. Unfortunately, no complete tubulin sequences have been published for goldfish so it was not possible to compare the degree of similarity between the parasite and host tubulin sequences.

Tubulin molecules are the structural building blocks of microtubules in eukaryotic cells. In trypanosomes, microtubules are critical for parasite survival because they are the major components of the cytoskeleton and flagellum, and play a role in cell division as the main structural components of the mitotic spindle [143]. Tubulin molecules are heterodimeric, consisting of an α subunit and a β subunit. The two molecules are similar in size (≈55 kDa) and are also similar in primary sequence. Although tubulin sequences have been relatively highly conserved throughout evolution, however, the tubulin molecules of some species of early eukaryotes, including the parasitic protozoa, possess different characteristics in terms of binding to tubulin ligands such as colchicine and taxol, and in terms of susceptibility to different chemotherapeutic agents, when compared to host tubulin [143, 189]. These observations, in addition to the fact that tubulin is one of the most abundant, if not most abundant, protein of the kinetoplastid parasites, have lead to the identification of protozoan tubulin as a target for chemotherapy and vaccine development. For example, tubulin is a known target of the benzimidazoles, which are used as antihelminthics in mammals [46]. Treatment with drugs that target tubulin results in the inhibition of microtubule formation in helminths but some members of this group are also known to have anti-protozoal activity, including anti-trypanosome effects *in vitro* [393]. Most importantly, these drugs are several hundred times more active against the protozoan parasites than the mammalian cells [270]. While trypanosomes often show differential susceptibility to different benzimidazole compounds, they show marked susceptibility to the vinca alkyloids. Members of this group include vinblastine and rhizoxin and these compounds are inhibitors of trypanosome and *Leishmania* tubulin polymerization *in vitro* [27, 394]. Despite the effectiveness of vinblastine against *T. brucei*, it was also found to inhibit tubulin polymerization in mammalian cells, and therefore does not represent a good target for chemotherapy [270]. Susceptibility to the vinblastine-related compound rhizoxin is associated with a mutation in the asparagine residue found at position 100 of the β-tubulin amino acid sequence [357]. Since this residue is conserved in the *T. danilewskyi* β-tubulin sequence, it is possible that fish trypanosomes may also be susceptible to rhizoxin. Although identification of biochemical targets for chemotherapy is useful for treatment of infection, problems can be encountered with development of drug resistance.

Trypanosome tubulin has been identified as a candidate molecule for vaccine development. Immunization could be used prophylactically to prevent infection and subsequent transmission of the infective stages of the parasite with less chance for the development of resistance. Immunization of mice with tubulin isolated from *T. brucei* induces antibody production and a protective immune response to subsequent infection with a number of different species of African trypanosomes [220]. Immunization of mice with purified rat tubulin does not result in antibody production nor does it confer protection to live parasite infection. These results suggest that trypanosome tubulin is relatively invariant between species (because the induced response confers resistance to multiple trypanosome species), and that it is sufficiently different from host tubulin to induce a specific antibody response that does not result in the induction of auto-reactive antibodies [220].

Since trypanosome tubulin was identified as an antigenic component of the ES products that stimulated a protective immune response in goldfish, the ability of recombinant *T. danilewskyi* α- and β-tubulin to elicit protective immunity in naïve hosts was assessed. The results of the immunization studies suggest that the resistance of goldfish to infection with *T. danilewskyi*, as evaluated by the determination of mean parasitemia and prevalence of infection, can be enhanced following immunization with the recombinant proteins in conjunction with FCA. In most cases, a decrease in the circulating numbers of parasites and prevalence of infection was observed within the first week of infection when fish were injected with endotoxin-free vaccine preparations. These results are similar to the characteristics of infection following immunization of goldfish with *T. danilewskyi* ES products +

FCA, supporting the hypothesis that tubulin is one of the immunogenic components of *T. danilewskyi* ES products.

In general, the protective effect of the endotoxin-free subunits was lost when fish were administered preparations of the recombinant proteins containing LPS suggesting that LPS negatively affects the immune response of the fish to the immunizing antigen. The United States Food and Drug Administration guidelines restrict the use of biological material containing more than 0.5 EU/mL in animal and human drug and vaccine testing [309]. The concentration of endotoxin in the samples of recombinant *T. danilewskyi* tubulin containing endotoxin was greater than 0.25 EU/mL; however, it was not toxic to the fish. Nevertheless, there appears to be an inhibitory effect of endotoxin on the generation of tubulin-induced protective immunity in immunized goldfish. At permissive concentrations, LPS initiates macrophage activation through binding of a receptor complex including TLR-4 [38]. Through the activation of macrophages, and induction of an inflammatory response, a Th1 cytokine profile is generated that results in the reciprocal down-regulation/inhibition of antibody responses. In small quantities, LPS can also act as a B-cell mitogen resulting in polyclonal activation of B-lymphocytes that produce antibodies that may or may not be reactive with parasite antigens [81]. Consequently, LPS can behave as an adjuvant in vaccines in which cell-mediated responses are critical for the resolution of infection. Alternatively, it may be inhibitory in systems in which a Th2-polarized antibody response is required. Analysis of lymphocyte activation in response to tubulin in the presence and absence of LPS would contribute to the identification of the mechanisms involved in the lack of protective immunity generated following immunization with recombinant tubulin in which LPS was not removed.

The source of the tubulin molecules in ES products is unknown but tubulin is likely released from parasites that are lysed during the preparation procedure. Alternatively, membrane-bound molecules possessing tubulin-like epitopes may be sloughed from the surface during incubation as part of the ES mixture. *In vivo*, lysis of developmental forms of the parasites that are not adapted to living in the vertebrate host would result in the exposure of intracellular components to the host immune system. A more in depth understanding of the biology of the vector forms of these parasites would enhance our understanding of the molecules that are required for survival and persistence of the bloodstream forms in the fish.

Limited proteolysis of mammalian tubulin has lead to the identification of major antigenic regions of the proteins corresponding to the C-termini of both α- (amino acids 415-430) and β-tubulin (amino acids 412-431) [79]. The C-terminal regions are also considered to be areas of flexibility that are exposed on the surface of microtubules that may be spontaneously released from the protein [79, 311]. If the C-termini of the *T. danilewskyi* tubulin subunits are also spontaneously released from the proteins, they may be responsible for the immunogenicity of the parasite ES products.

The plasma from goldfish that were immunized with endotoxin-free tubulin subunits, and the corresponding pre-immunization samples, contained antibodies that recognized recombinant α- and β-tubulin. Since goldfish appear to possess pre-existing antibodies that recognize *T. danilewskyi* tubulin, we are unable to determine whether anti-tubulin antibodies are also stimulated as a result of immunization. However, enhancement of natural antibodies to trypanosome tubulin has been reported following immunization with *T. cruzi* exoantigens, and furthermore, antibodies with specificity for additional tubulin epitopes were observed following *T. cruzi* infection in mice [141, 364]. Regardless of the nature of the anti-tubulin antibodies, the results of the studies performed with rabbit anti-recombinant α-tubulin IgG suggest that anti-tubulin antibodies have a negative impact on the survival of parasites *in vitro*. This effect was also demonstrated for *T. brucei* incubated with rabbit anti-*T. brucei* tubulin anti-serum [221]. Anti-tubulin antibodies, induced by administration of tubulin, recognize epitopes of the molecule that are different from those recognized by natural antibodies [234]. Proteolysis of the native *T. danilewskyi* tubulin molecules, or systematic deletion of amino acids from the expressed recombinant tubulin subunits, would be beneficial in the mapping of the epitopes recognized by natural versus induced anti-tubulin antibodies in goldfish.

In vitro, anti-tubulin antibodies prevent the polymerization of tubulin subunits to form microtubules [91]. Although the mechanism of action of anti-trypanosome tubulin antibodies is unknown, immunofluoresence studies suggest that the antibodies are taken up by the parasites, likely through the flagellar pocket [221]. The flagellar pocket is the only area of the parasite at which endocytosis and exocytosis occur [202]. The antibodies may be taken up specifically by receptor-mediated endocytosis or by pinocytosis. Previous research has suggested the existence of antibody receptors on the surface of *Leishmania* and trypanosomes that may become internalized in the flagellar pocket [61]. Directional movement of antibody bound to parasite antigen towards the flagellar pocket, followed by internalization of the complexes has been observed in *T. brucei* [315]. Putative functions for the kinetoplastid antibody receptors include facilitating entry into host cells by species with intracellular stages, and antigenic mimicry [61, 113, 382]. Anti-tubulin antibodies have also been shown to inhibit flagellar motility and division of eukaryotic cells [271-2]. Once the anti-tubulin antibodies are internalized by the trypanosomes, binding to cytosolic tubulin, microtubules of the sub-pellicular lattice, or tubulin involved in the formation of the mitotic spindle would undoubtedly alter the replication and motility of the parasites.

The second objective of my thesis work was to evaluate the possible mechanisms of antibody-mediated immunity to *T. danilewskyi* in goldfish. The importance of specific antibodies induced by exposure to foreign antigens has been well established in higher vertebrates, and they are known to have both direct and indirect effects on the control of infection by the host of a wide variety of pathogens. Mammalian antibodies play a role in direct agglutination of pathogens, inactivation of secreted or membrane bound virulence factors and toxins, and complement activation. In addition, they may add specificity to the antimicrobial activity of phagocytic and cytotoxic cells [200]. Many of the same activities have been reported for fish antibodies; however, it is also known that fish are limited in the efficacy of their antibody responses because most possess only one class of antibody. Furthermore, fish are unable to perform any of the antibody modifications that result in increased antibody affinity in mammals [397] and they are impaired in their ability to mount a true anamnestic response on secondary exposure to antigen. Teleost acquired immune responses are susceptible to fluctuations in temperature and they are sensitive to increased levels of stress hormones [45, 67, 183, 245, 318]. Consequently, the importance of innate immune mechanisms has become amplified in the immunological control of fish pathogens.

Among the most poorly studied pre-existing humoral immune mechanisms in fish are natural antibodies. In mammals, natural antibodies (mainly IgM) are produced by a subset of long-lived B-cells that produce germline encoded, unmutated immunoglobulins of broad specificity [64, 191]. Natural antibodies in mammals are reactive with both host and pathogen-derived T-independent antigens and are thought to act as part of the first line of defense against invading pathogens. Like the fish immunoglobulins, they are known to have limited mechanisms for diversification and affinity maturation. Natural antibodies have gained notoriety in mammalian systems because of their association with a number of autoimmune disorders but they have also been shown to be effective in the early control of microbial infections [30]. Anti-α-galactosyl antibodies have been found in the serum of healthy humans but may be enhanced during infection with *T. cruzi*. The anti-galactosyl antibodies participate in the complement-mediated lysis of bloodstream forms of *T. cruzi* [12]. Due to their reliance on innate mechanisms of host defense, natural antibodies may also represent an important immune surveillance mechanism in fish.

Preliminary evidence suggests that natural antibodies that cross-react with *T. danilewskyi* tubulin are present in the plasma of healthy goldfish. In addition, partially purified and purified IgM from non-infected, healthy goldfish dramatically inhibits the viability of the parasites *in vitro*. It seems that concentration of the IgM by purification is required to inhibit trypanosome viability *in vitro* since the same effect was not observed when the parasites were incubated with whole serum from naïve and infected hosts as part of the complement studies (Chapter 7).

It is not known whether the natural antibodies that react with *T. danilewskyi* tubulin participate in the inhibition of parasite growth; however, the ability of rabbit anti-*T. danilewskyi* tubulin IgG to inhibit parasite growth *in vitro* has been demonstrated. Pre-absorption of the purified IgM with *T. danilewskyi* tubulin would remove the anti-tubulin antibody pool and the results of this treatment on the trypanocidal activity of goldfish IgM could then be evaluated.

Paradoxically, the partially purified IgM isolated from fish during the acute stage of the infection, during which time fish harbor high levels of parasites, had diminished trypanocidal activity when compared to the partially purified IgM from non-infected fish. There are a number of possible explanations for this phenomenon including depletion of the effective antibodies by high concentrations of parasites, and parasite products, in circulation. Alternatively, or perhaps concomitantly, the parasites may induce immunosuppression that directly affects the natural antibody response of the host. Both *T. cruzi* and *T. brucei* are known to cause inhibition of lymphocyte responses during the acute phase of the infection that allows the parasites to establish long lasting infections [20, 247]. One of the mechanisms by which this occurs is polyclonal activation of lymphocytes by soluble and membrane bound trypanosome molecules. Production of large quantities of polyreactive IgM during *T. danilewskyi* infection would result in the dilution of the pool of effective anti-trypanosome antibodies. One example of a trypanosome polyclonal activator is the *T. cruzi trans*-sialidase that induces activation of lymphocytes and stimulates apoptosis in a variety of immune cell types [124, 209]. A gene encoding *trans*-sialidase from fish trypanosomes has been identified and *trans*-sialidase activity has been observed in crude *T. danilewskyi* cell lysates [3]. Since *trans*-sialidase is an important *T. cruzi* virulence factor, further characterization of the effects of the *T. danilewskyi* homologue on the cells of the goldfish immune system is warranted. An additional possibility for the reduction of natural antibodies following parasite infection is a physiological response, initiated by the host or induced by infection, involving a switch from the production of natural antibodies that play a role in non-specific immune surveillance, to production of parasite-specific antibodies that results in the enhanced efficacy of IgM later in the infection. The change in antibody production may be a result of changes in the fish B-cell population in response to alterations in the cytokine profile produced by other immune cells such as macrophages and T-cells. Unfortunately, the markers for the identification of fish T- and B-lymphocytes are poorly defined and to date no specialized populations, such as the natural antibody-producing $CD5^+$ B-cells in mammals, have been identified.

There are a number of possible sources for goldfish natural antibodies that have been well studied in mammals, but not as well studied in teleosts. The generation of natural antibodies in humans has been connected to stimulation by the hosts own gastrointestinal flora [203]. Likewise, immunoglobulins generated during exposure to other species of hemoflagellates may be cross-reactive with *T. danilewskyi* antigens. Natural antibodies may also be passed from mothers to their offspring and there is precedent for the detection of immunoglobulins and other nonspecific humoral factors in the eggs and larvae of sea bream immunized against *Photobacterium damsela* [152]. Regardless of the source of the natural anti-*T. danilewskyi* antibodies, the results of the Western blotting of recombinant tubulin subunits with pooled fish plasma suggest that individual fish likely have different levels of naturally occurring antibodies. Similarly, natural goldfish antibodies that react with *A. salmonicida* A-protein are present in variable levels within the population that correlate with the outcome of infection [340]. Since individual goldfish display variability in their response to *T. danilewskyi* infection it is likely that these observations are linked to the ability of individual fish to control the initial parasitemia, perhaps through the activity of pre-existing IgM. An additional benefit of the presence of natural antibodies to trypanosome tubulin in fish serum is that the B-cells that produce natural anti-tubulin IgM can also act as APCs that stimulate acquired immunity. The importance of T-cell mediated responses to tubulin in generating a protective immune response is also implied by the requirement for FCA in the *T. danilewskyi* ES product and recombinant tubulin subunit vaccine preparations.

Although the evidence strongly supports the existence of goldfish natural antibodies that might play a role in the protection of goldfish from *T. danilewskyi* infection, it is equally possible that other innate humoral effector molecules that purify with goldfish IgM are responsible for the anti-trypanosome activity. Small molecules such as antimicrobial peptides might contaminate the IgM preparations. Allthough antimicrobial peptides have activity against *T. cruzi* and *T. brucei in vitro* the effects of natural antibiotics on protozoan parasites from fish have not been studied [27, 146, 237]. Few antimicrobial peptides have been described from fish but they are known to have activity against bacterial, fungal, and protozoan pathogens of fish and mammals [69, 112, 204, 261-2]. In addition, other molecules such as lipids might co-purify with the IgM fraction following PEG precipitation and superose 6 purification that would not be detected using silver staining. Both lipid molecules and IgM are components of trypanosome lytic factor-2 (TLF-2), which is the main serum lytic factor associated with the resistance of humans and other primates to infection by *T. b. brucei* [303, 378]. Lipids and lipid-containing proteins can also act as reservoirs for some antimicrobial peptides [344]. The presence of these types of molecules in the partially purified and purified goldfish IgM fractions has not been ruled out.

Another mechanism of antibody-mediated immunity that has been demonstrated for protozoan infections in mammals and fish is fixation of complement and activation of the classical pathway of activation. The complement system is part of the innate branch of immunity but may also be activated in response to antibody-antigen complexes. Fish possess an elaborate complement system consisting of multiple isoforms of some central components, including C3, that have the ability to bind to different complement activating surfaces [352]. In addition, other fish hemoflagellates such as *C. salmositica* and *T. borreli* are susceptible to the classical pathway of complement activation [108, 317]. *T. brucei* and *T. cruzi* are both susceptible to complement-mediated lysis during the late acute and chronic phases of the infection in which immunosuppression is overcome and parasite specific complement-fixing antibodies are produced [44, 269]. The results of the studies involving the effects of goldfish complement on *T. danilewskyi in vitro* have shown that the trypanosomes are not normally susceptible to complement-mediated lysis by goldfish serum regardless of the presence of specific antibodies [296]. This result is not surprising since viruses, bacteria, protozoans, helminthes, and even tumor cells have evolved ways in which they avoid becoming targets of the complement system [10, 86, 134, 163, 181].

The mechanisms that lead to evasion of the complement system by pathogenic organisms fall into three categories. The first includes the presentation of non-complement activating surfaces to the immune system. In this category, tumor cells express high levels of complement regulatory molecules found on normal host cells that protect them from complement activity. These molecules include decay accelerating factor (DAF, CD55), membrane co-factor protein (MCP, CD46), and CD59 [134]. Other pathogens will bind soluble host complement regulatory factors including factor H that accelerate the breakdown of complement convertase enzymes [10, 244]. The presence of large quantities of sialic acid on the surfaces of cells (such as mammalian erythrocytes) will also stabilize the binding of factor H and prevent complement activity [243, 395].

The second category includes the use of soluble and membrane-bound proteases that attack and degrade complement components. The best example of a parasite the employs this strategy is *Leishmania*. The members of this genus possess a surface protease, gp63, which has been implicated in the invasion of host cells by promastigotes. The activity of gp63 has also been linked to the degradation of complement components bound to the surface of the parasites [53]. Although proteases similar to gp63 have been identified in other kinetoplastids including *T. brucei* their role in the evasion of complement by this species has not been fully elucidated [100]. Although protease activity have been observed for the fish kinetoplastid *C. salmositica* [418], no proteases or protease activity has been identified in *T. danilewskyi*, which would suggest that this mechanism might not be employed in the resistance of *T. danilewskyi* to complement.

The final category includes pathogens that employ antigenic mimicry to escape recognition by complement [409]. Viruses are the most well known for their ability to synthesize molecules that are related to the hosts' own complement regulatory molecules [35]. However, *T. cruzi* also possesses a GPI-anchored protein known as the complement regulatory protein (CRP) that is related to human DAF in structure and function and is known to contribute significantly to the resistance of the parasites to complement-mediated lysis [268].

The precise way(s) in which *T. danilewskyi* avoids the activity of goldfish complement is unknown; however, it is almost certainly related to a parasite surface protein. The parasites may fail to activate complement, or they may be bound by goldfish C3 and subsequently prevent formation of the convertase enzymes that lead to target cell lysis. We have shown that removal of the parasite surface molecules results in an increase in susceptibility of the trypanosomes to goldfish complement by the alternative pathway of activation. The parasites are able to regain resistance following a period of incubation of at least 6 hours provided that they are able to synthesize protein [296]. Similar results have also been obtained for enzymatic treatment of *T. cruzi* [335, 366]. Therefore, it seemed reasonable to hypothesize that *T. danilewskyi* might also possess a CRP-like molecule on the surface of the cells that prevents complement activity. Despite a number of different attempts to isolate such a molecule from a variety of *T. danilewskyi* preparations, none could be identified. Although these results do not preclude the existence of a complement-binding molecule on the surface of *T. danilewskyi*, it does suggest that another mechanism, such as the presence of a membrane-bound protease, may be involved. Generation of antibodies towards the *T. danilewskyi* molecules that prevent complement activity in the later phases of the infection may abrogate the effect of the protective molecules, rendering the trypanosomes susceptible to antibody-mediated complement lysis in an atypical manner.

9.2 Future directions

Future experiments should focus on the characteristics of the goldfish immune response, and of *T. danilewskyi,* which permit establishment of the parasites since the initial parasite levels have an impact on the outcome of the infection [390, 403]. In terms of the goldfish immune response, a more in-depth study of the innate immune response to the parasites is required since fish rely heavily on non-specific immune defenses, and since these are the components of the immune system that the parasites will first encounter. For instance, a detailed study of the profile of cytokine expression at the gene and protein levels following *T. danilewskyi* infection will lead to a better understanding of the type of acquired immune response that will be generated later in the infection. For example, it seems that *T. danilewskyi* does not induce macrophage activation in the classical manner [320]. It has been proposed that these trypanosomes may activate macrophages by an alternative route, which according to the mammalian paradigm, would result in a Th2-type response [180]. *T. danilewskyi* also proliferates in the presence of goldfish macrophage supernatants [42]. Induction of alternatively activated macrophages, that produce polyamines as a result of arginase-mediated metabolism of L-arginine, might represent an important parasite survival strategy. *T. brucei* and *T. cruzi* have been shown to use polyamines as a growth factor and both proliferate in the presence of alternatively activated macrophages [260]. The existence of classically and alternatively activated macrophages in fish could be supported by the evaluation of the expression of the arginase and iNOS enzymes, as wells as activating and deactivating cytokines, such as TNFα and TGFβ, in goldfish macrophages following exposure to *T. danilewskyi.* Changes in the expression of goldfish genes that are involved in the immune response to *T. danilewskyi* could be detected using suppressive subtractive hybridization of blood and kidney leukocyte mRNA from naive and infected hosts. Also, since *T. danilewskyi* is susceptible to NO *in vitro* [320], it would be interesting to determine the effects of incubation of parasites with macrophages that have been pre-activated in a classical manner by pro-inflammatory mediators on the survival of *T. danilewskyi.*

To further characterize the innate immune response of goldfish to *T. danilewskyi*, the repertoire of natural antibodies that react with parasite molecules should be examined. In several instances I have observed binding of antibodies from normal, healthy fish to proteins found in various parasite antigen preparations, including ES products. In light of our recent results it seems that natural antibodies to parasite antigens might play a role in the inhibition of parasite growth, at least *in vitro,* and some of these molecules could be identified using Western blotting with serum from uninfected fish. Since natural antibodies that react with *T. danilewskyi* tubulin have already been identified in the plasma of goldfish, and since rabbit anti-tubulin antibodies inhibit parasite growth *in vitro*, it would interesting to determine whether natural anti-tubulin antibodies are in part responsible for the effects of purified goldfish IgM on parasite growth *in vitro*. Also, the range of natural anti-tubulin antibody concentrations within a population of fish could be determined using an ELISA, and the susceptibility of fish with different levels of natural anti-tubulin antibodies to *T. danilewskyi* infection could be evaluated.

To determine whether molecules other than IgM are responsible for the inhibition of *T. danilewskyi in vitro*, lipid fractions could be removed from the IgM preparations, or the serum lipid fractions could be isolated by silica gel chromatography or KBr-flotation and tested for trypanocidal activity. Nothing is known about the effects of antimicrobial peptides on *T. danilewskyi* although they have been shown to cause death of *T. brucei* and *T. cruzi* trypomastigotes *in vitro* [27, 146, 237]. A few antimicrobial peptides have been identified in fish; however, the various fish genome and expression library sequencing projects will likely result in the identification of more antimicrobial peptide gene sequences. The effects of various mammalian cationic peptides on *T. danilewskyi in vitro* could also be evaluated.

Immunization of goldfish with recombinant α- and β-tubulin subunits resulted in an increase in resistance to infection with *T. danilewskyi*. Therefore, it seems likely that the natural anti-tubulin antibody pool is augmented by immunization. Since the whole native tubulin molecule was required to induce protection against *T. brucei* infection in mice, immunization of goldfish with a combination of recombinant α- and β-tubulin subunits, or an increased concentration of each, should be attempted to improve the efficacy of vaccination. Little is known about the mechanism of activity of anti-tubulin antibodies; however, it does not appear to involve agglutination of fish parasites. Evidence suggests that the antibodies bind to intracellular targets of *T. brucei* and that the antibodies are taken up by the parasites in the flagellar pocket [221]. Immunocytochemistry utilizing electron microscopy would aid in the localization of anti-tubulin antibodies to the flagellar pocket of *T. danilewskyi* and would also identify any intracellular targets.

T. danilewskyi is able to resist the effects of complement *in vitro* but the mechanism by which they accomplish this feat is unclear. The use of monoclonal antibodies and sera made deficient in certain complement components would facilitate the identification of the step(s) in the complement cascade inhibited by the parasites. Since it seems unlikely that a specific C3 binding molecule contributes to resistance, it would be possible to test for protease activity in parasite lysates. Inclusion of protease inhibitors in the assays involving exposure of trypanosomes to goldfish complement would lead to an increased susceptibility of the parasites to lysis if a protease were involved in complement resistance. The selective removal of specific carbohydrate side-chains from the mucin-like glycoproteins that coat the entire trypanosome might also improve the access of complement to the parasite cell surface.

9.3 Conclusion

In conclusion, the work presented in this thesis has identified *T. danilewskyi* tubulin as a component of the immunogenic ES products of the parasites. I was able to successfully clone, sequence, and express both the α- and β-tubulin subunits in a prokaryotic expression system and the recombinant molecules were used to immunize goldfish. The results of the immunization studies suggest that parasite tubulin subunits can increase the resistance of goldfish to *T. danilewskyi* infection. This is the first report of the immunogenicity of trypanosome tubulin in fish.

Rabbit anti-tubulin antibodies inhibit the survival of parasites *in vitro* by an unknown mechanism that likely requires internalization of the tubulin-specific IgG. Similarly, purified goldfish natural antibodies have also been shown to inhibit parasite growth *in vitro*. It remains to be determined whether goldfish natural anti-tubulin antibodies contribute to the inhibition of parasite growth *in vitro*, and whether variability in the levels of the antibodies results in differential susceptibility to infection. These studies represent the first description of the direct activity of parasite-specific antibodies on the viability of *T. danilewskyi*.

T. danilewskyi was also found to be resistant to complement-mediated lysis *in vitro*. The mechanism of resistance is unknown but it is related to the ability of the parasites to synthesize new surface proteins following enzyme treatment. Resistance to complement is a common immune-avoidance mechanism of blood-borne pathogens and contributes to their ability to establish long-term infections in vertebrate hosts.

It is clear that *T. danilewskyi* and their goldfish hosts have developed a relationship in which the parasites ensure establishment of chronic infections that are crucial for transmission while in most cases preventing the death of the host. Although this relationship may be perceived as a lack of pathogenicity of the parasites, under conditions of stress that are known to affect a number of innate and acquired immune mechanisms in fish, the infection has been associated with morbidity and mortality related to levels of circulating parasites and susceptibility to secondary infection. Therefore, further characterization of the immunological relationship between these two organisms is required to determine how the parasites establish persistent infections, and how the teleost immune system is able to control and eliminate *T. danilewskyi* under normal conditions. The results will not only have direct implications for the control of trypanosomiasis in fish, but will also advance our understanding of the early vertebrate immune system and trypanosome biology.

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